Targeting ErbB receptors as anticancer therapy: factors of resistance and sensitivity

Janmaat, M.L.

2006

Targeting ErbB receptors as anticancer therapy: Factors of sensitivity and resistance

Maarten Janmaat

The research described in this thesis was performed at the Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands.

The printing of this thesis was financially supported by J.E. Jurriaanse Stichting, Dr.Ir. Van de Laar Stichting, Pharmamar, and AstraZeneca.

Printed by PrintPartners Ipskamp

ISBN 90 865 902 76

VRIJE UNIVERSITEIT

Targeting ErbB receptors as anticancer therapy: Factors of sensitivity and resistance

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. L.M. Bouter,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Geneeskunde
op woensdag 11 oktober om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Maarten Laurens Janmaat

geboren te Bladel en Netersel

promotor: prof.dr. G. Giaccone

copromotor: dr. J.A. Rodriguez

In order to give the true science of
the movement of the birds in the air
it is necessary first to give the science
of the winds, and this we shall prove by
means of the movements of the water

Leonardo Da Vinci

Aan Karin en Julie

Contents

Chapter 1

INTRODUCTION

1. The epidermal growth factor receptor pathway

The growth and proliferation of mammalian cells is a tightly regulated process that is modulated by stimuli from their environment. Epidermal growth factor (EGF)-related peptides represent a class of molecules that can trigger cell proliferation, and several other cellular processes such as differentiation, migration, and survival. Binding of EGF-like peptides to the epidermal growth factor receptor (EGFR) at the cell surface leads to a cascade of intracellular reactions that transduce signals to the nucleus, resulting in particular gene expression patterns. In various tumour types the regulation of EGFR activity is altered, due to increased or aberrant expression of the receptor or its ligands. EGFR deregulation contributes to many aspects of carcinogenesis. Several strategies have been developed that specifically target the EGFR and inhibit its activity in tumour cells. In particular, small-molecule EGFR tyrosine kinase inhibitors and antagonistic EGFR-specific monoclonal antibodies represent two promising classes of anti-cancer agents.

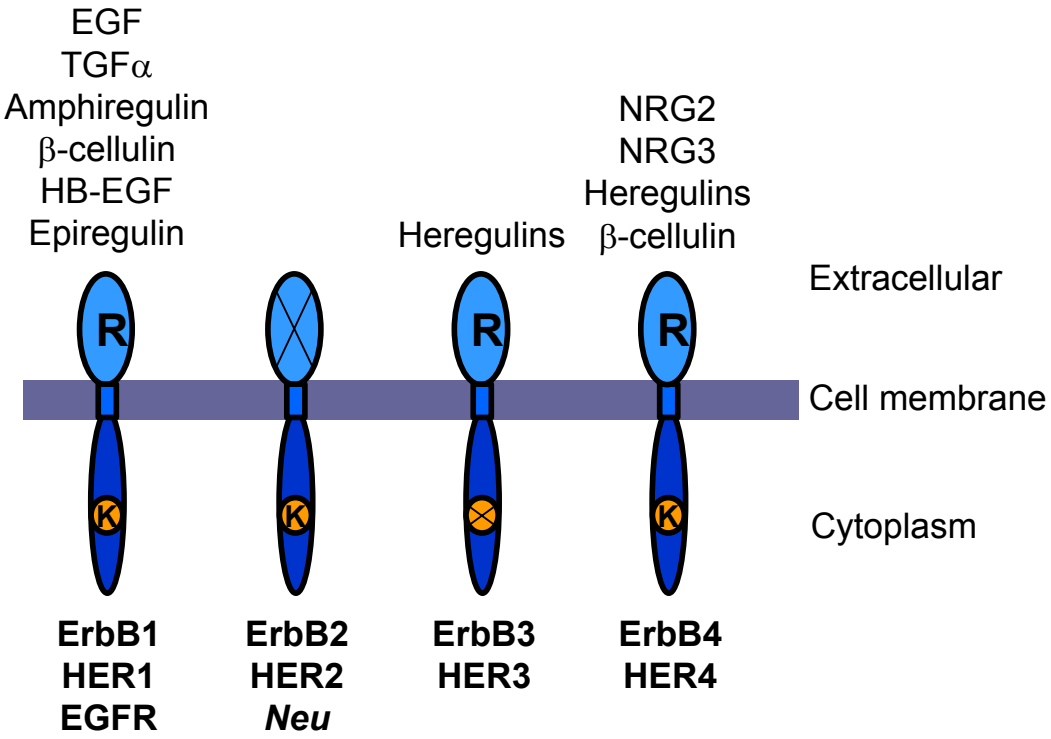


Figure 1. ErbB receptors and their ligands. Schematic protein stuctures of the four ErbB receptors localised on the cell membrane. On top are the ligands indicated that bind to the receptors. Note that no ligands have been identified that bind ErbB2, and that ErbB3 lacks a tyrosine kinase domain. R, receptor domain; K, tyrosine kinase domain.

1.1 Regulated activation of EGFR involves a network of receptors and ligands

EGFR, also referred to as ErbB1 or HER1, is a 170-kDa, transmembrane protein, consisting of an extracellular ligand-binding domain, a short transmembrane region and an intracellular domain containing protein tyrosine kinase activity. Three other ErbB proteins that are structurally and functionally related to EGFR have been identified: ErbB2/HER2/Neu, ErbB3/HER3 and ErbB4/HER4. Together, the ErbB receptors belong to the class I of receptor tyrosine kinases (RTKs; Figure 1). ErbB receptors exist as inactive monomers in the plasma membrane, and dimerise upon ligand binding, either as homodimers or heterodimers. A number of EGF-related peptides specifically bind to the ErbB1, -3, and -4 receptors, whereas no ligand for ErbB2 has been identified so far (Figure 1). However, ErbB2 is the preferred heterodimerisation partner of all other ErbB proteins [1-3], suggesting that ErbB2 principally functions as co-receptor. In addition, heterodimers containing ErbB2 are more stable and transduce signals that are more potent than other receptor dimers [4-6]. Each ligand has specific binding properties, resulting in the formation of particular dimer combinations [7]. However, in tumour cells in which ErbB2 is overexpressed, heterodimerisation with ErbB2 leads to receptor activation by a broader panel of ligands [8]. In addition, biochemical properties of the ligand-receptor interaction determine the signal strength and duration, as well as the fate of the receptor, which is either degradation in lysosomes or recycling. For instance, EGF binding targets the receptor primarily to the lysosomes (Figure 2), whereas the receptor is recycled to the cell membrane when TGFα is bound as ligand [3,9]. Thus, ligand diversity and specificity, as well as the variety of ErbB dimer-combinations and properties, regulate ErbB signalling at the input level.

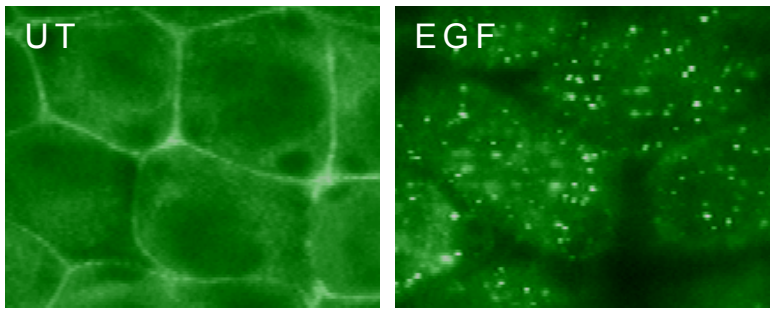


Figure 2. EGF induces internalisation of EGFR. EGFR localization in H460 lung cancer cells was visualized with immunofluorescence. Cells were either untreated (UT, left panel) or stimulated with EGF for 10 minutes (EGF, right panel).

1.2 EGFR activates various intracellular signalling pathways

Ligand binding drives dimerisation, intracellular kinase activation, and subsequent trans-autophosphorylation of specific tyrosine residues located in the C-terminal region of the ErbB proteins. ErbB3 lacks a functional kinase domain [10], and therefore can only be phosphorylated when dimerised with one of the other ErbB receptors. The phospho-tyrosines then serve as docking sites for adapter molecules such as SHC and Grb2, kinases as PI3K and Src, or negative regulators of receptor activity, like the tyrosine phosphatases SHP1 and 2 [11]. Each ErbB receptor displays a distinct pattern of autophosphorylation sites that bind different effector proteins, although considerable redundancy has been reported [12]. The ligand identity as well as the dimer partner, determines which tyrosines are phosphorylated, and hence which proteins are recruited [6]. Primarily, the mitogen activated protein kinase (MAPK) pathways are activated by all ErbB family members [13] (Figure 3). In contrast, ErbB3 most efficiently activates PI3K, as this receptor harbours six tyrosine residues that can recruit the p85 subunit of PI3K when phosphorylated [14]. PI3K does not directly bind to EGFR, although the PI3K pathway can be activated by EGFR via effector molecules such as Grb2 [15]. Signal transducers and activators of transcription (STAT) family members are usually recruited to phospho-tyrosine sites of cytokine receptors, activated by JAK kinases, and translocated to the nucleus where they function as transcription factors [16]. In contrast to JAK-mediated STAT activation by cytokine receptors, STAT proteins are constitutively associated with the inactive EGFR and are activated upon ligand stimulation independently from JAK kinase activity [17]. The Src family of kinases is another class of downstream substrates of EGFR and is thought to contribute to the activation of numerous downstream molecules, including Ras and PI3K [18]. Conversely, Src has been demonstrated to phosphorylate EGFR on Tyr845 [18], although the significance of Src-mediated EGFR phosphorylation is not well understood. In addition, phospho-lipase Cγ (PLCγ) specifically binds and is activated by EGFR [19], which results in the activation of the protein kinase C (PKC) pathway (Figure 3). Finally, Eps15 and Cbl are specific substrates of EGFR [20,21] that have functions in receptor downregulation [22,23].

Ultimately, the kinase signalling pathways connect EGFR to transcription factors in the nucleus (Figure 3), resulting in regulated transcriptional activation.

In addition to activation of gene expression, the EGFR pathway directly interferes with cellular processes as protein synthesis, cell survival, and cell motility [24-26].

Alternative to the activation of signalling cascades, the EGF receptor may directly function as a transcription factor in the nucleus [27]. As the EGFR lacks a DNA-binding domain, EGFR-mediated transcriptional activity requires the (co) factor STAT3 [28].

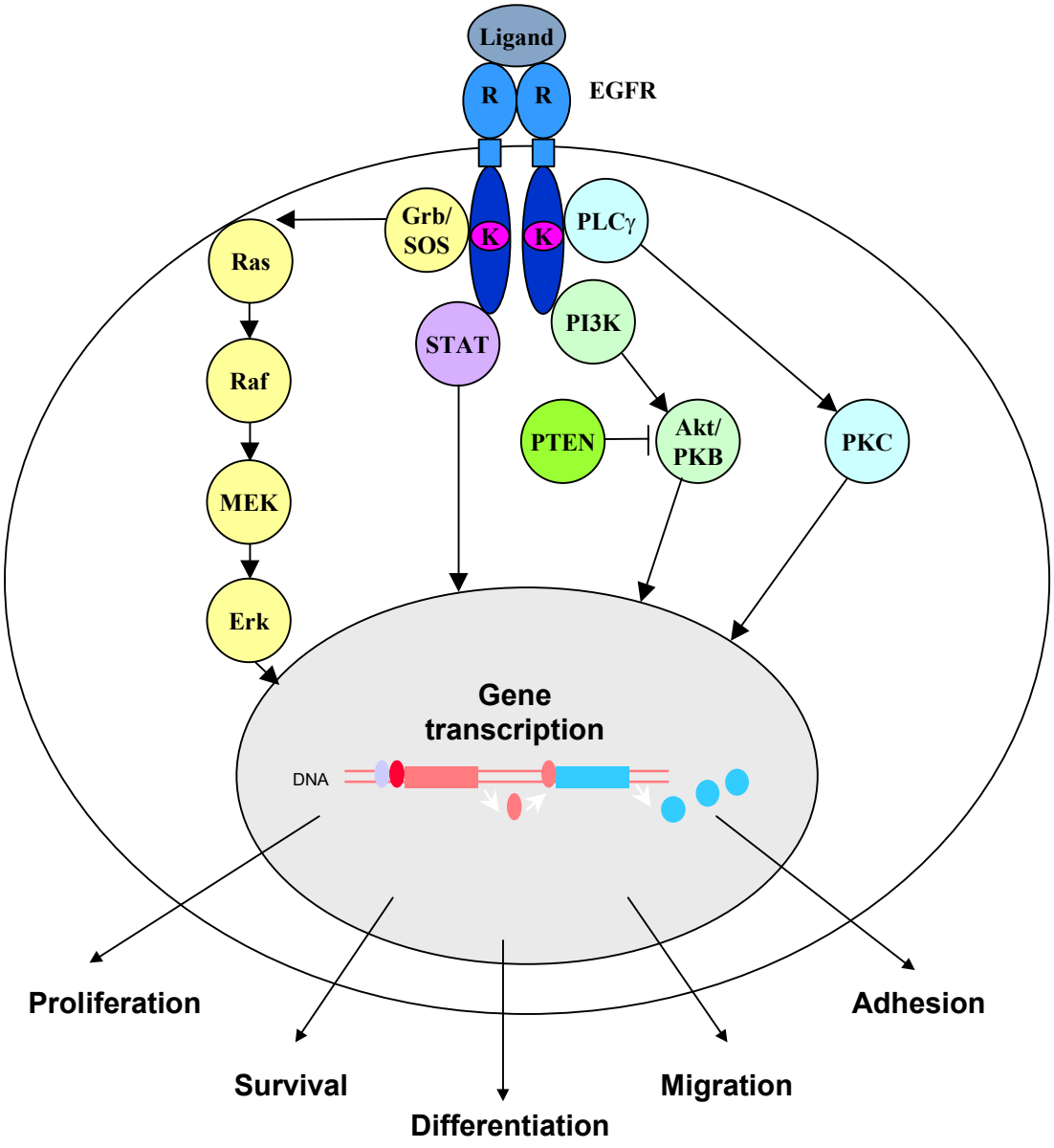


Figure 3. The EGFR signalling pathway. Activation of the EGFR induces downstream activation of several intracellular signalling pathways. The pathways ultimately converge in the nucleus, where gene transcription is activated and subsequently cellular responses are initiated. See the text for further details.

1.3 Activation of the EGFR pathway can lead to a range of responses

The EGFR pathway controls distinct biological processes and the actual physiological response depends on the particular ligand, the ErbB dimer and the downstream signalling pathways activated [29,30], as well as the cellular context [13].

EGFR activity is critically involved in embryonic development [31]. Experiments with genetically modified mice have shown the requirement for the EGF receptor and the other ErbB family members in development, as null mutations in any of the ErbB receptors are lethal [32-37]. More specific analysis of embryos lacking the *EGFR* gene, revealed that EGFR is involved in organogenesis of many meso- and ectodermal derived organs, including brain, heart and lung [32-34,38]. In addition to embryogenesis, ErbB receptors have roles in the adult organism, in particular in the development of the mammary gland [39].

1.4 Trans-activation of EGFR

It has been recently recognised that, in addition to EGF-related peptides, other stimuli that do not directly bind the receptor, such as hormones, lymphokines, and stress factors, can activate EGFR [40]. From experiments using dominant-negative EGFR mutants and specific kinase inhibitors, it has become clear that this trans-activation of EGFR is essential for several, mainly mitogenic, responses induced by various stimuli [40]. In contrast to the classical ligand-induced activation and autophosphorylation of EGFR, trans-activation of the receptor is generally mediated by other protein kinases. Agonists of a range of G-protein coupled receptors (GPCR), including thrombin, lysophosphatic acid (LPA), and endothelin-1 have been demonstrated to induce mitogenic responses via EGFR [41]. Trans-activation of EGFR by GPCRs is thought to be mainly mediated via c-Src tyrosine kinases [42], although GPCRs have also been demonstrated to activate matrix metalloproteinases involved in the cleavage of the EGFR-ligand proHB-EGF, thereby stimulating EGFR [43]. Other stimuli that trans-activate EGFR include cytokines, via activation of the tyrosine kinase JAK2 [44]; cell adhesion through aggregation of integrin receptors [45,46]; membrane depolarisation, involving c-Src activation [42]; stress-stimuli such as heat-shock [47], hyperosmotic conditions [48] or UV-irradiation [49]; and insulin-like growth factor (IGF) [50].

Table 1. EGFR expression in different tumor types and the frequency (%) of studies showing an association between EGFR expression and overall survival.

Tumor type	Proportion of tumors expressing EGFR (%)	% of studies showing an association between EGFR expression and overall survival
Bladder	72 [51]	63 (*n*=11) [52]
Breast	14–91 [51]	55 (*n*=11) [52]
Cervical	33-72 [53-57]	67 (*n*=6) [53,55-59]
Colorectal	25–77 [51]	67 (*n*=3) [52]
Glioma	40–63 [51]	n.e.
Head and neck	43–100 [51]	82 (*n*=11) [52]
NSCLC	32–84 [51]	10 (*n*=10) [52]
Ovarian	35–70 [51]	67 (*n*=9) [52]
Pancreatic	30–95 [51]	n.e.
Renal	50–93 [51]	n.e.
Oesophageal	39-71 [60-62]	69 (*n*=13) [52]
Gastric	2-34 [63-65]	50 (*n*=6) [52]
Endometrial	43-82 [66-70]	40 (*n*=5) [52]

1.5 Increased and aberrant EGFR expression is associated with oncogenesis

Accumulation of genetic alterations or changes in the expression of genes involved in growth, survival and DNA repair, contribute to the development of cancer [71]. In this regard, high expression of EGFR is frequent in many types of cancer, including tumours of the aerodigestive tract, breast, ovary and brain (Table 1). However, the expression levels between and within tumour types demonstrate considerable variation. Several explanations for the high variability have been put forward, including differences in the methods used for the measurement of EGFR expression, different detection thresholds, and heterogeneity of EGFR expression within tumours (reviewed in [51]). A review of the literature by Nicholson and colleagues revealed that EGFR expression is a strong prognostic factor in tumours of the head and neck, ovary, cervix, bladder, and esophagus [52]. In contrast, it has only a modest or poor prognostic significance in gastric, breast, endometrial, colorectal and non-small cell lung (NSCL) carcinomas [52]. The true prognostic significance of EGFR could have been underestimated, since there was substantial diversity between the studied patient populations, in addition to the above-mentioned variability between detection methods used in the different studies. Nevertheless, several recent studies, including a meta-analysis considering 2185 patients, confirmed that

EGFR overexpression or high gene copy number were not significant prognostic factors for the overall survival of NSCLC patients [72,73].

Importantly, many tumours co-express EGFR and its ligands, particularly TGFα, which correlates with poor patient outcome [12,74] and points to autocrine signalling as an important force for EGFR-driven tumor growth. In addition, over-expression of other ErbB members, in particular ErbB2, and transactivation by heterologous signalling networks can result in increased EGFR activity.

Alternative to increased expression, a significant proportion of tumours contain a mutated *EGFR* gene, which most commonly translates in constitutively active or hyperactive forms of the receptor. A high percentage of gliomas (57-86%) express a truncated EGFR that lacks 267 amino acids from its extracellular domain (EGFRvIII), due to gene rearrangements that are often associated with gene amplification [75,76]. Although two immunohistochemical reports suggest that the EGFRvIII mutation occurs in several other tumour types including non-small cell lung cancer (NSCLC) [75,77], detailed mutational studies recently revealed that such mutations are rare or absent in NSCLC [78-80]. In contrast to the large deletions detected mainly in gliomas, somatic *EGFR* mutations were identified in the kinase domain of the receptor in a fraction of NSCLCs [78-80]. Several different point mutations and small deletions were identified in exons 18 to 21 of the *EGFR* gene, encoding the kinase domain. These mutations are thought to translate in a receptor that is more potent in transducing growth and survival signals [79,81]. Importantly, several studies indicate that the presence of *EGFR* kinase domain mutations strongly correlates with a dramatic clinical response to EGFR tyrosine kinase inhibitors (TKIs) in NSCLC patients [78-80]. In addition to NSCLCs, *EGFR* kinase domain mutations have been identified at very low frequency in squamous cell carcinoma of the head and neck and colorectal tumours [82,83].

Altogether, the observations that aberrant EGFR activity, due to EGFR overexpression or mutations, is implicated in oncogenesis point to EGFR as a potential target for anti-cancer therapy.

2. Inhibition of the epidermal growth factor receptor as anticancer therapy

2.1 The EGFR as target for anticancer therapeutics

It has become clear that the EGFR pathway has a role in the development and progression of cancer, since increased or aberrant expression of the receptor and/or its ligands is frequent in many types of tumours and correlates with a more aggressive disease and poor prognosis [84,85].

This provides a rationale for the inhibition of the EGFR as anticancer therapy [86]. Indeed, many agents have been developed that specifically target the receptor, ranging from toxin-conjugated anti-EGFR antibodies or ligands to antisense oligo-nucleotides [87,88] (Figure 4). Among these, monoclonal antibodies (mAbs) directed against the extracellular domain of the receptor and small-molecule tyrosine kinase inhibitors are the most advanced EGFR-targeted agents in clinical development (Table 2), and will be discussed below.

Table 2. Clinical development of ErbB inhibitors.

Agent	Characteristic	Target	Tumor type	Stage
Gefitinib	Reversible TKI	EGFR	NSCLC	Marketed
Erlotinib	Reversible TKI	EGFR	NSCLC, pancreas	Marketed
Lapatinib	Reversible TKI	EGFR and ErbB2	Breast	Phase III
CI-1033	Irreversible TKI	Pan-ErbB	NSCLC, breast, ovary	Phase II
EKB-569	Irreversible TKI	EGFR	Colon, SCLC	Phase II
ZD-6474	TKI	EGFR and VEGFR-2	NSCLC	Phase II, ongoing
AEE788	Reversible TKI	EGFR, ErbB2, VEGFR-2	NSCLC	Phase II, ongoing
BMS-599626	Reversible TKI	EGFR and ErbB2	-	Phase I
Cetuximab	Chimeric mAb	EGFR	Colon, H&N, NSCLC, pancreas	Marketed, Phase III (NSCLC)
ABX-EGF	Human mAb	EGFR	Colon, renal	Phase III
MDX214	Human mAb	EGFR	NSCLC	Phase II, ongoing
EMD-7200	Humanised mAb	EGFR	H&N, ovarian, colon, cervix	Phase II
h-R3	Humanised mAb	EGFR	H&N	Phase II
Pertuzumab	Humanised mAb	ErbB2	Breast, ovarian, prostate, NSCLC	Phase II
Trastuzumab	Humanised mAb	ErbB2	Breast	Marketed
EGF vaccin	Vaccin	EGF	NSCLC	Phase II

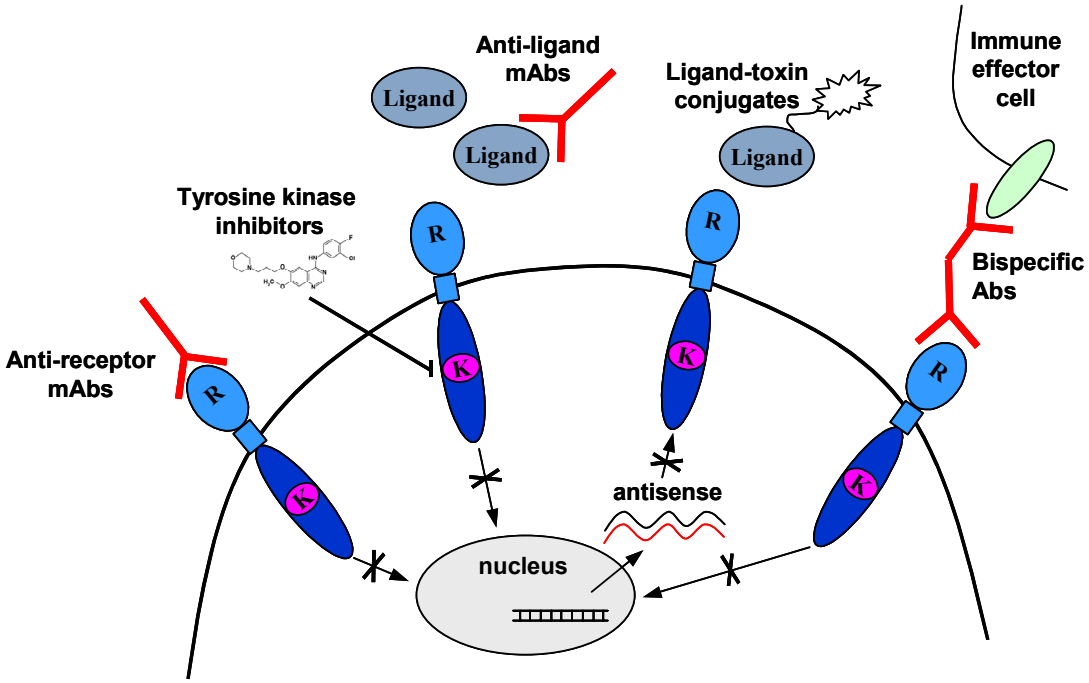


Figure 4. EGFR targeting agents and their sites of action.

2.2 Preclinical studies with monoclonal antibodies

In the early 1980s, Mendelsohn was the first to propose the use of antagonistic EGFR-specific monoclonal antibodies as anticancer therapy [86,89,90]. Mendelsohn´s group has generated two mouse mAbs directed against the EGFR (mAb 528 and mAb 225) with similar affinity for EGFR as EGF and TGFα. These antibodies compete with the natural ligands for receptor binding, thereby preventing receptor activation and promoting receptor internalisation [91-93]. The antibodies inhibit the *in vitro* and *in vivo* growth of human cancer cells derived from breast, prostate, lung, bladder, pancreas, head and neck, and colon tumours [86,89,90]. To prevent an immunological human anti-mouse response that may compromise the therapeutic efficacy of mAb 225 when repeatedly administered to humans, a chimeric human-mouse mAb 225 (C225 or cetuximab) has been developed by replacing the murine constant region of the immunoglobulin with a human IgG1 constant region. Cetuximab has a 10-fold higher affinity for EGFR than mAb 225, and is more effective at inhibiting *in vivo* tumour growth [94]. In preclinical models, mAb 225 as well as cetuximab enhance the anti-tumour activity of chemotherapeutic agents, such as cisplatin and doxorubicin, as well as radiotherapy [95-98].

Another promising anti-EGFR antibody is ABX-EGF (or mAb E7.6.3), which is a fully human IgG2 mAb with high affinity for EGFR. ABX-EGF has been shown to eradicate established A431 xenografts in nude mice [99]. In addition to antibodies directed against wt-EGFR, a panel of mAbs specifically recognising the type III mutated receptor (EGFRvIII) has been developed [100], from which the murine mAb Y10 has been proved to be the most active *in vitro* and *in vivo* [101-103]. The mechanisms underlying the activity of the anti-EGFR mAbs 225/cetuximab are the most extensively studied, and will be discussed in more detail below.

2.3 Mechanisms of action of mAb 225/cetuximab

Several molecular and biological mechanisms have been implicated in the anti-tumour activity of mAb 225/cetuximab [86,89,90]. Anti-EGFR mAbs specifically inhibit ligand-induced EGFR tyrosine kinase activation, proving the inhibition of EGFR activation as direct mechanism of action. Treatment with mAb 225/cetuximab results in inhibition of tumour cell growth *in vitro* as well as *in vivo* [91,93,94,104].

The growth inhibition induced by anti-EGFR mAbs is often associated with an arrest of cells in the G1 phase of the cell cycle. This has been shown in non-transformed cells [91,105] and in cultured malignant cells [106-108]. *In vitro* studies demonstrated that the molecular mechanism underlying this G1 phase arrest involves upregulation of the CDK2 inhibitor $p27^{kip1}$ [108-110], which was confirmed *in vivo* using mice with xenografts derived from a human bladder tumour cell line [111]. In addition, inhibition of the Erk and Akt kinase signalling pathways, which lie more directly downstream of EGFR, was associated with antitumor activity of cetuximab *in vitro* and *in vivo* [112,113], suggesting that the inhibition of Erk and/or Akt may serve as surrogate markers for EGFR inhibition. In addition, persistent activity of the Erk and/or Akt pathways may contribute to resistance to cetuximab [113].

Cytotoxity has only rarely been observed in cells treated with the anti-EGFR mAbs. However, mAb 225 induces apoptosis (programmed cell death) in the DiFi colon adenocarcinoma cell line, initiated by caspase-8 and followed by activation of caspase-3 and –9, resulting in the death of all cells within 48 hours [107,114]. A less efficient induction of apoptosis has also been observed in cell lines

derived from head and neck, breast and vulval tumours (e.g. A431) when treated with cetuximab as single agent [97,113,115]. In contrast, we have not observed any induction of apoptosis in cetuximab-treated non-small cell lung cancer cell lines [113]. Several observations in sensitive cells support the idea that Bcl-2 family members are involved in regulating cetuximab-induced apoptosis. First, the mitochondrial, anti-apoptotic protein Bcl-2 is downregulated or inactivated upon cetuximab-treatment [97,115], whereas the sensitive DiFi cells lack detectable Bcl-2 expression [116]. Moreover, transfection studies in our laboratory demonstrate that A431 cells overexpressing exogenous Bcl-2 were protected from cetuximab-induced apoptosis [113]. In contrast, the protein levels of the pro-apoptotic Bcl-2 family member Bax are upregulated in several tumour cell lines upon cetuximab-treatment [97,116]. These studies thus demonstrate that treatment with anti-EGFR mAbs favours pro-apoptotic mechanisms involving Bcl-2 protein family members, which lead only in a subset of cell lines to the activation of apoptosis, suggesting that the threshold to undergo apoptosis is not reached in most cells by the inhibition of EGFR.

Studies with mAb 225 or cetuximab in combination with chemotherapy or radiotherapy demonstrated synergistic anti-tumour activity in a range of pre-clinical models. Combination studies with mAb 225 and cisplatin or doxorubicin were among the first to demonstrate augmented antitumor activity in human breast and vulval cell xenografts [95,96]. In addition, later reports demonstrated *in vivo* synergistic anti-tumour activity between mAb 225 or cetuximab and subtoxic doses of paclitaxel [117], topotecan [118], and gemcitabine [38]. Although the exact mechanism for this synergism is unclear, it is known that growth factors can activate pro-survival signals, so inhibition of these survival signals by mAbs may lower the threshold to induce cell death, as discussed above. More recently, cetuximab has been shown to enhance the anti-tumour effect of radiation therapy in cultured tumour cells as well as in tumour cell xenografts [97,98,119]. In addition to inhibition of pro-survival signalling, potential mechanisms contributing to the enhanced radiosensitivity are inhibition of cell proliferation, resulting in reduced repopulation after radiotherapy, and inhibition of the DNA-repair machinery (reviewed in [120]). Anti-EGFR mAbs have also been shown to induce enhanced anti-tumour activity in combination with specific inhibitors of

other molecules involved in EGFR signalling, including anti-ErbB2 antibodies [119,120], farnesyl-transferase inhibitors [121], and inhibitors of protein kinase A [122].

Several mechanisms that may play a role *in vivo* but not in the cell culture setting, such as the inhibition of angiogenesis, invasion and metastasis, as well as the activation of immune responses, have been suggested to contribute to the anti-tumour activity of cetuximab. The anti-angiogenesis effect of cetuximab has been proposed to be the result of the reduced secretion of angiogenesis factors by tumour cells, including VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor) and IL-8 (interleukin 8), which has been correlated with a decreased amount of new blood vessels [38,111,123]. Perrotte *et al.* have also demonstrated that cetuximab-treatment of mice bearing human bladder carcinoma xenografts, prevents metastasis to lymph nodes and lungs [111], suggesting the reduction of tumour cell migration by cetuximab. Although immune responses can be mediated via the human IgG1 part of cetuximab, these are not essential for the anti-tumour effect of cetuximab *in vivo*, as a truncated fragment of cetuximab lacking the human IgG1 portion was still be able to inhibit xenograft growth, albeit less efficiently [95]. Thus, several mechanisms contribute to the additional anti-tumour effect of cetuximab in *vivo*, including inhibition of angiogenesis and metastasis, and possibly a minor induction of an immune response.

Based on the promising preclinical data, many clinical studies have been performed, some of which have been completed. Cetuximab was shown to have clinically significant activity when given alone or in combination with irinotecan in patients with irinotecan-refractory metastatic colorectal cancer [124]. On the other hand, the addition of cetuximab to high dose radiation in patients with advanced squamous cell carcinoma of the head and neck demonstrated a statistically significant prolongation in overall survival [125]. Based on the results of these studies, cetuximab has been registered for the treatment of irinotecan-refractory colorectal cancer, and will be registered shortly for the treatment of head and neck cancer in combination with radiotherapy.

2.4 Preclinical studies with EGFR tyrosine kinase inhibitors

The finding that engineered mutations in the adenosine triphosphate (ATP)-binding site of the EGFR disable ligand-induced responses [126,127], indicated that this part of the receptor is essential for EGFR tyrosine kinase activity and downstream signalling. In order to specifically inhibit EGFR kinase activity, hundreds of natural and synthetic compounds were screened searching for molecules that compete with ATP for EGFR-binding. Many compounds of different chemical classes that effectively inhibit EGFR kinase activity have been identified, some of which are in advanced clinical development (Table 1). These molecules differ in their ability to bind the EGFR ATP-binding pocket -either reversibly or irreversibly- or in the capacity to additionally inhibit other members of the ErbB family of receptors [128]. Among the agents that have been investigated in preclinical tests, gefitinib (ZD1839 or IRESSA™) and erlotinib (OSI-774 or Tarceva™) have been most extensively studied [129]. A review of preclinical studies with EGFR-TKIs is given below, while an overview of clinical studies with EGFR-TKIs will be given in section 3 of this chapter.

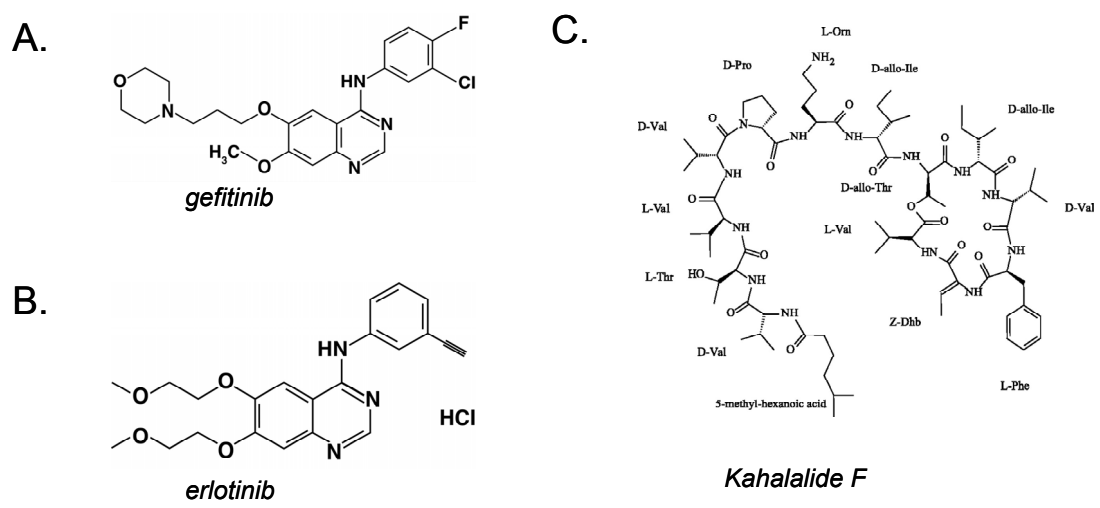


Figure 5. Chemical structures of the ErbB targeting agents.

2.4.1 Gefitinib

Gefitinib is an orally active, selective, and reversible EGFR-TKI that chemically belongs to the class of anilinoquinazolines [129] (Figure 5). Similar to EGFR-blocking monoclonal antibodies [86,89,90], gefitinib induces mainly cytostatic effects *in vitro* [130,131], although cytotoxic effects have been observed in a few cases [50,113,132]. It has been suggested that gefitinib, like cetuximab, favours

several pro-apoptotic mechanisms involving Bcl-2 family members, as the pro-apoptotic protein BAD is activated by gefitinib in breast cancer cells [50], whereas overexpression of the apoptosis-suppressor Bcl-2 reverts gefitinib-induced cell death of A431 cells [113].

Gefitinib is active against a wide variety of tumour cell lines [113,130-135]. As the EGFR is part of a large signalling network [13], the sensitivity of cells to gefitinib is likely to be affected by multiple cellular factors. EGFR expression levels were initially found to be unrelated to response to gefitinib, since xenografts expressing high, moderate, and low amounts of EGFR showed growth inhibition upon gefitinib treatment [130,131]. Subsequent studies, however, showed that cell lines with high EGFR expression levels were sensitive for gefitinib [135]. In contrast to expression levels, the activity status of EGFR determined by the presence of *EGFR* mutations and heterodimerisation with other ErbB family members may be a more important predictor of gefitinib sensitivity (reviewed in [136]).

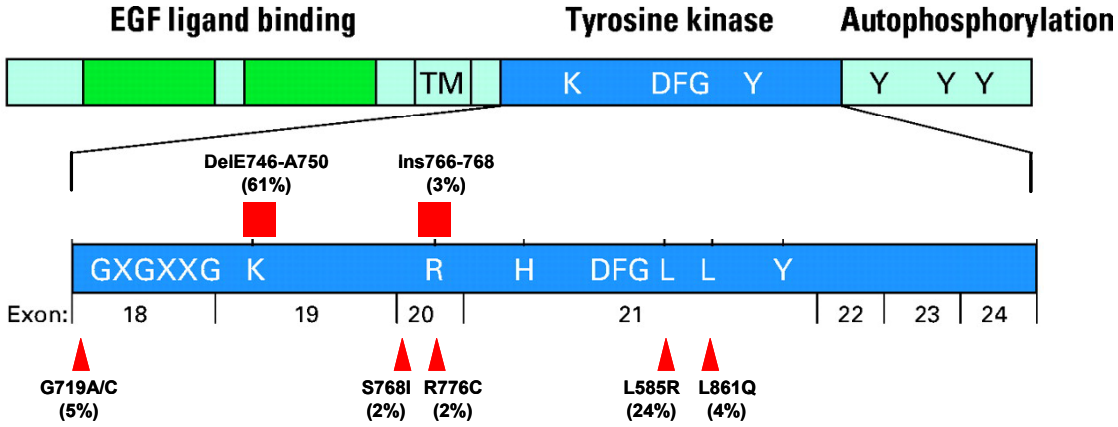


Figure 6. EGFR gene structure and localization of mutations in hotspot regions of the tyrosine kinase domain.

In 2004, two simultaneous publications demonstrated that somatic mutations in the kinase domain-encoding region of the *EGFR* gene were closely associated with a dramatic response to gefitinib in NSCLC patients [78,79]. So far, three classes of mutations have been identified: missense mutations, deletions, and in frame insertions (Figure 6). Functional analysis in fibroblasts transfected with two different mutant forms of EGFR showed that activation of mutant EGFR is more intense and prolonged compared with the wild-type receptor [81]. Moreover, *in vitro* data show that cells expressing mutant EGFR preferentially

activate the Akt and STAT anti-apoptotic pathways, and that treatment of these cells with gefitinib resulted in rapid apoptotic cell death [81].

On the other hand, cells expressing high levels of ErbB2 have also been shown to be particularly sensitive to gefitinib [132,134,135]. At least 100-fold higher concentrations gefitinib are required for the direct inhibition of ErbB2 compared to EGFR [137], but gefitinib may indirectly inhibit ErbB2 activity by sequestering ErbB2 through the induction of signalling-inactive EGFR-ErbB2 heterodimers [138,139]. In addition to ErbB2, ErbB3 is implicated in gefitinib-sensitivity, as ErbB3 couples EGFR to the PI3K/Akt pathway in gefitinib-sensitive NSCLC cell lines [140].

It is well-established that proteins that are involved in cell cycle progression, in particular $p27^{kip1}$ and cyclin D, play an essential role in the G1-phase cell cycle arrest induced by gefitinib [132,141]. Moreover, we and others have suggested that intrinsic and persistent activity of kinase pathways downstream of EGFR, such as the Ras/Raf/MEK/Erk and the PI3K/Akt pathways, may provide a mechanism of resistance to gefitinib [113,133,134,142-144]. EGFR-independent activity of EGFR downstream pathways may be the result of activating mutations in downstream molecules such as *k-ras* and *PI3K* [145,146] or depletion of the tumour suppressor *PTEN* [147]. Indeed, we and others have shown that mutations in *k-ras* were associated with resistance of NSCLC cells to gefitinib [148,149]. In line with this, depletion of PTEN by siRNA resulted in EGFR-independent activity of PI3K/Akt signalling and resistance to gefitinib [143]. Conversely, resistance to gefitinib in PTEN-null HER-overexpressing tumour cells can be overcome through restoration of PTEN function or pharmacologic modulation of constitutive PI3K/Akt pathway signalling [142].

Finally, in addition to defects within the pathway, EGFR-independent signalling and associated resistance to gefitinib can be a result of increased activation of these pathways via other growth factor or hormone receptors, such as IGF-1R [150].

Gefitinib has been combined with a variety of cytotoxic agents. Except in the case of gemcitabine, the combinations resulted in enhanced antitumor effects in cultured cells and *in vivo* models [130,131]. In addition, gefitinib-treatment resulted in synergistic effects in combination with radiation [151-153]. As in de case of the EGFR-specific antibodies, the underlying mechanism of additive or

synergistic effects of gefitinib and chemo- or radiotherapy is thought to involve inhibition of EGFR-mediated survival signals, and the decrease of the apoptotic threshold. Moreover, it has been suggested that gefitinib directly inhibits the multidrug resistant pump BCRP (or ABCG2), resulting in enhanced activity of antitumor drugs in BCRP-expressing cells [154-158]. Importantly, sequence-dependent effects were reported in cells treated with combinations of gefitinib with radiation or chemotherapy (cisplatin and/or 5-FU). The best results were achieved when gefitinib was applied before radiation and before or during chemotherapeutic treatment, whereas an antagonistic effect was observed when gefitinib was applied after cytotoxic treatment [151]. These data indicate the need to be cautious in the design of clinical trials using combinations of gefitinib and cytotoxic agents.

A relatively new development is the preclinical evaluation of treatment with gefitinib combined with other novel, biological agents, such as the ErbB2 monoclonal antibody trastuzumab (Herceptin™) [132,159]. ErbB2 is the preferred dimerisation partner for EGFR, and EGFR/ErbB2 dimers are thought to induce more potent signals than other EGFR-containing dimers [1,2]. Simultaneous inhibition of EGFR and ErbB2 resulted in additive or synergistic effects in ErbB2-overexpressing breast cancer cells [132,159]. These preclinical data prompted a currently ongoing phase II trial combining trastuzumab and gefitinib in breast cancer patients [160]. Pharmacologic modulation of constitutive Ras/Erk or PI3K/Akt pathway signalling in combination with gefitinib treatment also resulted in enhanced cytotoxic effects [142,148].

In addition to the often limited, antiproliferative effects observed *in vitro*, several mechanisms of action that are only active *in vivo*, such as inhibition of angiogenesis and invasion and metastasis, have been attributed to gefitinib [137,153,161-163]. The anti-angiogenesis effect of gefitinib has been proposed to be the result of reduced secretion of pro-angiogenesis factors [161,162], while gefitinib can also directly inhibit the growth and cell-cell interactions of endothelial cells [137,137,153,162]. Furthermore, combination of gefitinib with cytotoxic treatment, such as paclitaxel or radiation, potentiated the anti-angiogenesis effect [153,161].

2.4.2 Erlotinib

Erlotinib is an EGFR-specific quinazoline derivative (Figure 4) that inhibits the activity of EGFR in cell-free assays and in intact cells at nanomolar concentrations (IC50 values of 2 nM and 20 nM, respectively). An initial report showed that erlotinib induces apoptosis and growth inhibition in several tumour cell lines *in vitro*, which was associated with the induction of $p27^{kip1}$ expression and a blockade in the G1-phase of the cell cycle [164]. Moreover, erlotinib has a substantial effect on the growth of human head- and neck-, and pancreatic derived-derived xenografts growing in athymic mice, which was associated with a decrease in phosphorylation of Erk, but not of Akt [165,166]. In skin and derived biopsy specimens, phosphorylated forms of EGFR, Akt, and Erk were decreased after treatment with erlotinib [167,168]. Synergistic effects were observed when erlotinib was combined with cisplatin, doxorubicin, gemcitabine, and other cytotoxic agents in preclinical models [165,169]. Moreover, erlotinib selectively inhibited molecular effectors such as extracellular matrix components, metalloproteases, and serine proteases that are involved in the invasion of human glioblastoma cell lines expressing the EGFRvIII mutant [170]. This mutant receptor cannot be recognized by EGFR-specific monoclonal antibodies as cetuximab, illustrating one of the advantages of EGFR-TKIs over monoclonal antibodies. Erlotinib is currently being tested in advanced clinical trials in various human malignancies (Table 2).

2.4.3 Other small molecule EGFR-TKIs

Many other small molecule inhibitors some of which inhibit other ErbB kinases in addition to EGFR have been investigated in preclinical studies. Several of these inhibitors are currently being tested in the clinic and will be briefly discussed here.

PD-183805 and its water-soluble analogue canertinib (CI1033), are irreversible inhibitors of all ErbB receptors (Table 2). These agents exhibit *in vitro* and *in vivo* antitumor activity as single agent, and in combination with cytotoxic drugs in several tumour models [171-174]. Synergistic apoptotic responses were found when cells were treated with combinations of canertinib and gemcitabine [174]. In this study, inhibition of the Erk and Akt kinase pathways was noted, similar

to other EGFR-targeted agents, and concurrent activation of the p38 stress pathway was reported, together contributing to the apoptotic response.

PKI166 is a dual EGFR/ErbB2 inhibitor that induces growth inhibition in several tumour-derived cell lines expressing high levels of EGFR and/or ErbB2 [175], and exhibits anti-tumour activity against several human tumour models in mice, commonly associated with anti-angiogenesis and anti-invasion effects [176-180]. Interestingly, when compared with an EGFR-specific kinase inhibitor (CGP-59326), PKI-166 was more efficient to inhibit the *in vitro* growth of tumour cells in the presence of EGF-related ligands [175], suggesting more pronounced anti-tumour effects of inhibitors that target both EGFR and ErbB2 compared to EGFR-specific agents.

Similar results have been published with several other small-molecule EGFR tyrosine kinase inhibitors in preclinical studies. Of note, lapatinib (GW572016), a novel dual EGFR/ErbB2 kinase inhibitor that is currently being tested in the clinic, has been demonstrated to be active against *in vitro* and *in vivo* human tumour models [181,182]. Finally, EKB-569 is an irreversible inhibitor of EGFR activity that inhibits potently the growth of cells overexpressing EGFR or ErbB2, but has little effect on cells with low expression levels of these receptors [183,184].

2.5 Kahalalide F

Kahalalide F (KF) is a novel antitumor agent that was originally isolated from the Hawaiian marine mollusk *Elysia rufescens* [185,186]. KF is chemically unrelated to the small-molecule EGFR-TKIs described above (Figure 5), but reports suggested that this compound inhibits EGFR and/or ErbB2 activity and reduces the expression of TGFα [187,188], although ectopic overexpression of ErbB2 did not protect against KF-induced cell death [189]. In addition to EGFR and ErbB2, lysosomes seem to be intracellular targets of KF [189,190]. In this thesis, we show that KF-induced cytotoxicity does not involve inhibition of EGFR or ErbB2, but involves downregulation of ErbB3 and downstream inhibition of Akt signalling [191].

KF has high cytotoxic activity against cell lines and tumour specimens derived from various human solid tumours, including prostate, breast, non–small-cell lung, ovarian, and colon carcinomas [188,192-195]. Moreover, KF has shown

antitumor activity against human prostate cancer xenografts in mouse models [193]. In contrast, nontumoral cell lines were 5 to 40 times less sensitive to KF [189], and bone marrow progenitors were not affected when treated with suprapharmacological concentrations of KF [196]. In a phase I clinical trial in solid tumours, antitumor activity was noted in patients harbouring hepatoma, melanoma, and breast and pancreatic carcinoma [197], and the activity of KF is currently being investigated in phase II clinical trials in patients with melanoma, hepatic carcinoma, and NSCLC [192].

3. Clinical studies with EGFR tyrosine kinase inhibitors

3.1 Gefitinib

The results of three phase I studies in patients with advanced solid tumours have been published [198-200]. Gefitinib was one of the first targeted agents that have been tested in the clinic and the goals were clearly different from trials with cytotoxic agents. Instead of the maximum tolerated dose, one goal was to determine the optimal biological dose. Other goals of the study were to establish pharmaco-kinetic and –dynamic parameters. On the other hand, the fact that expression of the EGFR is common in solid tumours, and the preclinical evidence available, suggested that gefitinib might have a broad antitumor activity. This led to the inclusion of multiple tumour types and no selection of patients based on tumour EGFR expression. Across the dose range tested (50-1000 mg/day), the most frequent adverse events were dose-dependent acneiform skin rash and grade 1 or 2 diarrhea. The latter was dose limiting, being severe and frequent in patients that received doses over 600 mg/day. Other toxicities that have been observed include nausea and transient and asymptotic transaminitis. All of these side effects were manageable and reversible on cessation of treatment.

To evaluate the effect of gefitinib on EGFR-TK activity, biopsies of the skin, which is known to express EGFR, were obtained before and after 28 days of treatment. Several known, EGFR-dependent molecular markers and downstream effects on proliferation were evaluated. These studies showed inhibition of EGFR-regulated signalling in patients treated with gefitinib, consistent with the proposed mechanism of action. Thus, downregulation of activated EGFR, MAPK,

and the proliferation marker Ki67 and upregulation of $p27^{kip1}$, phosphorylated STAT3, and apoptotic cells was observed after gefitinib treatment [199,201]. In these phase I studies, some antitumor activity was observed in patients with NSCLC, head and neck, ovarian, colorectal, prostate and breast cancers [198-200,202].

The promising phase I results prompted the rapid initiation of phase II studies in pre-treated patients with NSCLC, hormone-refractory prostate cancer, advanced breast cancer, advanced colorectal cancer, head and neck cancer, esophageal cancer, ovarian cancer and glioblastomas, amongst others. Currently, data are available of two large randomised phase II studies in pre-treated NSCLC randomised patients to 250 mg or 500 mg daily dose of gefitinib, doses that achieved sufficient blood concentrations to inhibit EGFR activation in preclinical models. One study (IDEAL1) enrolled 210 patients who received one prior chemotherapy regimen, which included a platinum drug [203]. The response rate was 18.5%, while approximately 40% of patients experienced symptom improvement. The other study (IDEAL2) enrolled 216 patients who received 2 or more regimens, which included a platinum drug and docetaxel. In this study, the response rate was 10% [204]. In both studies, withdrawals and grade III-IV adverse events were more frequent in patients receiving the higher dose, while the response rate was comparable, indicating that activity is achieved at a dose of 250 mg, and higher doses do not generate better efficacy but lead to enhanced toxicity [203,204]. Preliminary data of phase II trials in other tumour types indicate that gefitinib is generally well tolerated in combination with several cytotoxic therapies, and promising results have been observed in a monotherapy trial in recurrent or metastatic head and neck cancer [205], and in patients with advanced breast cancer [206].

At the same time, two very large randomised studies have been concluded with gefitinib and combination chemotherapy, and final results have been presented [207,208]. In these studies (INTACT1/2), chemo-naïve patients with advanced NSCLC were randomised to receive chemotherapy with placebo or 2 different doses of gefitinib (250 or 500 mg). Chemotherapy consisted of carboplatin/paclitaxel (INTACT2), which is standard in North America, or cisplatin/gemcitabine (INTACT1), which is more frequently employed in Europe. These studies confirmed the safety and tolerability of gefitinib, but failed to reach the major endpoint which was improvement in survival by the addition of

gefitinib, or any of the other endpoints, which included progression-free survival, time to worsening of symptoms, objective tumour response, and quality of life. Results of a phase III trial (ISEL) in which gefitinib was given as monotherapy to patients with advanced NSCLC have been presented recently. Gefitinib failed to prolong survival compared with placebo in NSCLC patients who had failed one or more lines of chemotherapy [209]. These clinical studies were robust and it was concluded that these efficacy results are definitive.

Thus, gefitinib has been registered for second and third line therapy of advanced NSCLC in Japan and several other countries. However, gefitinib failed to improve overall survival of NSCLC patients when given as second or third line therapy [209]. Therefore, the registration of gefitinib has been pulled back in the US and will be never given in Europe.

3.2 Erlotinib

Erlotinib is structurally related to gefitinib and, as observed in clinical studies, has a very similar toxicity and safety profile, with skin rash and diarrhea as dose-limiting toxicities. Promising activity has been seen in phase I trials, with reports of some complete responses [210-212]. The dose level chosen for further evaluation after phase I studies, is 150 mg daily, which is a dose just below the maximum tolerated dose of 200 mg/day. To date, the results of three monotherapy phase II trials have been reported. In a study with 56 NSCLC patients, 7 patients (11%) achieved a partial response, while 19 patients (34 %) had stable disease [213]. The response rate in NSCLC is similar to that obtained with gefitinib, indicating a consistent pattern of activity of this class of agents in NSCLC. Promising phase II results have also been reported for advanced ovarian cancer, refractory head and neck cancer, and hepatocellular cancer with response rates of 8.8%, 13%, and 7.9% respectively and disease stabilization in 44%, 29% and 59% of the patients [214-217]. Many phase I/II studies in various malignant diseases are currently being carried out with erlotinib in combination with several chemotherapeutics and/or radiation (reviewed in [218]). In these studies, responses have been reported for NSCLC, penile carcinoma, head and neck cancer and mesothelioma, while prolonged disease stabilization was observed in NSCLC, mesothelioma, head and neck, bladder, ovary, stomach and skin cancer [211,219,220].

Two large international studies of erlotinib in first-line treatment of advanced NSCLC in combination with chemotherapy versus chemotherapy alone have been concluded. These studies used the same chemotherapy regimens used in the gefitinib phase III studies (carboplatin/paclitaxel ['TRIBUTE' study] and cisplatin/gemcitabine ['TALENT' study]). Analogous to the studies with gefitinib, erlotinib with chemotherapy did not confer a survival advantage over chemotherapy alone [221,222]. Nonetheless, it is encouraging that in the 'TRIBUTE' study never-smokers seemed to experience an improvement in survival [222], which will be further investigated in future trials.

Recently, the results of a phase III trial in which erlotinib monotherapy was evaluated in NSCLC patients after failure of first- or second-line chemotherapy have been published (BR.21 trial). Compared to the placebo group, erlotinib treatment resulted in prolonged survival and a higher response rate [223]. The response rate was 8.9 percent in the erlotinib group compared to less than 1 percent in the placebo group ($P<0.001$). Progression-free and overall survival significantly improved from 1.8 to 2.2 months and from 4.7 to 6.7 months, respectively, in favor of erlotinib. In addition to NSCLC, erlotinib significantly improved survival and progression free survival in a phase III trial in advanced pancreatic cancer when combined with gemcitabine [224]. Based on the results of these studies, erlotinib has been registered for second and third line treatment of NSCLC and will be registered for the treatment of pancreatic cancer in combination with gemcitabine.

3.3 Determinants of sensitivity to gefitinib and erlotinib in NSCLC patients

Several factors have been put forward as potential cause for the disappointing results of the randomised clinical studies with gefitinib. One factor may be the lack of patient selection, which may have diluted a possible beneficial effect of the addition of gefinitib to combination chemotherapy. On the other hand, several reports suggest that expression levels of EGFR or ErbB2 do not correlate with response or survival in gefitinib-treated NSCLC patients [225,226]. In contrast, some clinical parameters have been associated with gefitinib sensitivity, including female gender, never-smoker status, adenocarcinoma histology and Asian ethnicity [204,227]. The major breakthrough in the

understanding of gefitinib responsiveness, however, came with the identification of somatic *EGFR* kinase domain mutations [78-80]. After the first excitement about retrospective reports that predicted over 80% response rate in *EGFR* mutant NSCLC patients [78-80], the enthusiasm is somewhat tempered as recent reports showed only a 46-60% response rates in *EGFR*-mutant patients with no benefit on overall survival [225,228]. In the same reports, *EGFR* amplification has been suggested to be an effective predictor for gefitinib efficacy and survival in NSCLC patients.

In line with the results with gefitinib, the presence of *EGFR* mutations has initially been associated with responsiveness to erlotinib [80]. However, mutational analysis of tumors from the 'TRIBUTE' and BR.21 trials showed that erlotinib-treated patients with mutant *EGFR* had a similar overall survival compared to the wild-type group, although the response rate was somewhat higher in the *EGFR* mutant group [229,230]. Investigators are now focusing on other possible markers of erlotinib efficacy, including EGFR expression, gene amplification, and activation of downstream molecules such as Akt. Similar to gefitinib, *EGFR* gene copy number may be an important predictor of response to erlotinib [230]. Conversely, the presence of *k-ras* mutations was associated with poorer clinical outcomes in patients treated with erlotinib and chemotherapy [229], indicating that *k-ras* mutations may predict resistance to erlotinib. Moreover, clinical features that were predictive for treatment efficacy include never-smoking status, female gender, adenocarcinoma histology, and Asian ethnicity, similar to gefitinib [223]. Moreover, the intensity of skin rash has been correlated with survival in a retrospective analysis [231]. The impact of *EGFR* mutations, amplification, and expression levels of ErbB receptors on gefitinib and erlotinib sensitivity are currently the subject of intensive investigation.

Besides the primary resistance related to the presence of *k-ras* mutations or EGFR-independent activity of Erk or Akt pathways, resistance to erlotinib or gefitinib eventually develops in most NSCLC patients who initially responded to treatment with these EGFR-TKIs. Two groups showed that acquired resistance to treatment with gefitinib or erlotinib was associated with a secondary point mutation in the EGFR tyrosine kinase domain in some cases, leading to the substitution of Threonine 790 to Methionine [232,233]. On the other hand, two reports from Asia demonstrated that the T790M mutation associated with EGFR-

TKI resistance can be present at diagnosis [234,235]. Interestingly, Kobayashi *et al.* showed that treatment with the irreversible EGFR-TKI CL-387,785 can overcome resistance of cell lines expressing the resistant EGFR mutant [236]. In addition to CL-387,785, the broad spectrum ErbB TKIs EKB-549 and CI-1033 were identified in an *in vitro* screening to have activity against cells expressing the resistant mutant [237]. These data thus suggest that second-generation EGFR-TKIs can be of importance in the treatment of NSCLC.

3.4 Other small molecules

CI-1033 is an irreversible inhibitor of all three ErbB members that function as tyrosine kinase (ErbB3 lacks a kinase domain). The data of several phase I studies testing different administration schedules have been presented. All schedules were generally well tolerated. Side effects include the ones observed with the other small molecules, with diarrhea and skin rash as dose limiting toxicities. In addition, some cases of thrombocytopenia and allergy have been reported [238]. Unfortunately, no complete or partial responses are reported in phase I trials, although several patients achieved stable disease [239]. Phase II studies are currently under way, including a randomized phase II trial in patients with advanced NSCLC, who failed prior platinum-based chemotherapy. In this study, patients are selected by having at least one of the four ErbB receptors positive by immunohistochemistry.

Phase I studies with the dual EGFR/ErbB2 inhibitor PKI-166 have been recently concluded. The major toxicities were similar to gefitinib and erlotinib, but an apparently higher incidence of liver toxicity has been noted with this agent [240]. Because of the liver toxicity the further development of PKI-166 has been discontinued.

The first phase I/II data with the dual EGFR/ErbB2 kinase inhibitor GW-572016 reveal that the compound is well tolerated in patients at concentrations up to 1250 mg per day as monotherapy, as well as in combination with chemotherapeutics [241-248]. In addition to skin rash and diarrhea, headache was one of the most common adverse events. In one study, the inhibition of activated Akt, activated Erk and cyclin D protein was associated with tumor cell apoptosis and regression of metastasis, and predicted for favorable clinical response [245]. Interestingly, two breast cancer patients that were resistant for

treatment with the anti-ErbB2 antibody trastuzumab had objective responses [245], while two gefitinib-resistant NSCLC patients achieved minor responses [244]. These observations suggest that the dual specificity of GW-572016 may be more effective in some patients compared to more specific agents. Phase II trials are ongoing, including in patients with trastuzumab refractory metastatic breast cancer and metastatic colorectal cancer [241,242].

EKB-569 is an irreversible EGFR-specific TKI, which inhibits the growth of tumour cell lines that overexpress EGFR or ErbB2 *in vitro* and *in vivo* [183]. The preliminary data of three phase I studies have been presented, in which several treatment schedules were tested as monotherapy in patients with advanced-stage solid tumours [249], and in combination with cytotoxic agents in patients with advanced pancreatic [250] and colorectal cancer [251]. Again, the results showed mild diarrhea and skin rash as major toxicities, indicating that EKB-569 is generally well tolerated.

In summary, the mechanism of action of the small molecule EGFR-TKIs involves the direct inhibition of EGFR activity and/or other ErbB members. The presence of mutations in the kinase domain-encoding region of the *EGFR* gene and *EGFR* gene amplifications have been strongly associated with sensitivity to EGFR-TKIs. In addition, the expression of ErbB2 or ErbB3 are markers of EGFR-TKI sensitivity, while expression levels of EGFR may be of importance in *EGFR* wild-type cells. High expression of p-Akt, in particular in EGFR positive cells, has been associated with gefitinib sensitivity of NSCLC patients, whereas *k-Ras* mutations may predict for resistance to EGFR-TKIs. Effective inhibition of downstream Ras/Erk and PI3K/Akt kinase pathways has been linked to the antiproliferative and –sometimes- pro-apoptotic effects of the EGFR-TKIs. Upregulation $p27^{kip1}$, downstream of Akt, appears to be essential for the growth delay induced by these agents. EGFR-TKIs have been shown to be potent inhibitors *in vivo* of tumour growth, anti-angiogenesis agents and to inhibit invasion and metastasis of tumour cells when given as single drug or in combination with cytotoxic treatment.

4. Outline of the thesis

The Introduction (*Chapter 1*) describes the epidermal growth factor receptor (EGFR) pathway and its role in embryonic development and cancer. The mechanisms of EGFR activation, and the downstream molecular and cellular effects are discussed. An overview is given of the status of ErbB-targeted therapies in preclinical and clinical development. In this thesis we have investigated the molecular mechanisms of ErbB-targeting anti-tumour agents, and examined several biological markers that may predict the outcome of cancer patients treated with such agents.

In *Chapter 2* we have investigated the molecular and cellular mechanism of action of EGFR inhibitors. Regarding the promising results of early clinical trials with the EGFR-TKI gefitinib in NSCLC patients, we were mainly interested in the effects of gefitinib in NSCLC-derived cell lines, and we compared gefitinib with the EGFR-specific antibody cetuximab. Since we observed that persistent, EGFR-independent activity of the downstream PI3K/Akt and/or Ras/Erk kinase pathways was associated with resistance to EGFR inhibitors, we tested the hypothesis described in *Chapter 3* that combining gefitinib with agents specifically inhibiting these downstream molecules may result in enhanced anti-proliferative or cytotoxic effects.

Chapter 4 describes the results of a clinical study of gefitinib monotherapy in previously treated, advanced esophageal cancer patients. Although the response rate was disappointing (2.8%), a considerable proportion of patients experienced stable disease (27.8%). We attempted to identify the patients that had benefit from gefitinib treatment, and for that purpose, we analyzed tumor material for several biological features. Although no EGFR mutations were detected, the patient outcome was significantly better in patients demonstrating high EGFR immunohistochemical expression. Conversely, k-ras mutations were found in two patients with progressive disease. In addition, squamous cell carcinoma histology or female gender predicted for better patient outcome.

In *Chapter 5* we investigated the molecular mechanism of action of the novel, marine-derived anti-tumor agent Kahalalide F (KF). Although this compound is chemically and functionally unrelated to EGFR-TKIs, we show that

KF-induced cytotoxicity involves downregulation of the EGFR-related receptor ErbB3 and downstream inhibition of Akt activity.

Finally, the results presented in this thesis and their implications in the clinic are discussed in *Chapter 6*, and directions for possible future research are specified.

5. References

1. Graus-Porta, D *et al.* ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J* **16**, 1647-1655 (1997).

2. Karunagaran, D *et al.* ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO J* **15**, 254-264 (1996).

3. Waterman, H *et al.* Alternative intracellular routing of ErbB receptors may determine signaling potency. *J Biol Chem* **273**, 13819-13827 (1998).

4. Beerli, RR *et al.* Neu differentiation factor activation of ErbB-3 and ErbB-4 is cell specific and displays a differential requirement for ErbB-2. *Mol Cell Biol* **15**, 6496-6505 (1995).

5. Graus-Porta, D *et al.* Single-chain antibody-mediated intracellular retention of ErbB-2 impairs Neu differentiation factor and epidermal growth factor signaling. *Mol Cell Biol* **15**, 1182-1191 (1995).

6. Olayioye, MA *et al.* ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner. *Mol Cell Biol* **18**, 5042-5051 (1998).

7. Tzahar, E *et al.* Bivalence of EGF-like ligands drives the ErbB signaling network. *EMBO J* **16**, 4938-4950 (1997).

8. Jones, JT *et al.* Binding specificities and affinities of egf domains for ErbB receptors. *FEBS Lett* **447**, 227-231 (1999).

9. French, AR *et al.* Intracellular trafficking of epidermal growth factor family ligands is directly influenced by the pH sensitivity of the receptor/ligand interaction. *J Biol Chem* **270**, 4334-4340 (1995).

10. Guy, PM *et al.* Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc Natl Acad Sci U S A* **91**, 8132-8136 (1994).

11. Wu, CJ *et al.* Inhibition of EGFR-mediated phosphoinositide-3-OH kinase (PI3-K) signaling and glioblastoma phenotype by signal-regulatory proteins (SIRPs). *Oncogene* **19**, 3999-4010 (2000).

12. Olayioye, MA *et al.* The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J* **19**, 3159-3167 (2000).

13. Yarden, Y *et al.* Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2**, 127-137 (2001).

14. Prigent, SA *et al.* Identification of c-erbB-3 binding sites for phosphatidylinositol 3'-kinase and SHC using an EGF receptor/c-erbB-3 chimera. *EMBO J* **13**, 2831-2841 (1994).

15. Wang, XY *et al.* Interleukin-11 induces complex formation of Grb2, Fyn, and JAK2 in 3T3L1 cells. *J Biol Chem* **270**, 27999-28002 (1995).

16. Imada, K *et al.* The Jak-STAT pathway. *Mol Immunol* **37**, 1-11 (2000).

17. Haura, EB *et al.* Mechanisms of disease: Insights into the emerging role of signal transducers and activators of transcription in cancer. *Nat Clin Pract Oncol* **2**, 315-324 (2005).

18. Parsons, SJ *et al.* Src family kinases, key regulators of signal transduction. *Oncogene* **23**, 7906-7909 (2004).

19. Chattopadhyay, A *et al.* The role of individual SH2 domains in mediating association of phospholipase C-gamma1 with the activated EGF receptor. *J Biol Chem* **274**, 26091-26097 (1999).

20. Fazioli, F *et al.* eps15, a novel tyrosine kinase substrate, exhibits transforming activity. *Mol Cell Biol* **13**, 5814-5828 (1993).

21. Levkowitz, G *et al.* Coupling of the c-Cbl protooncogene product to ErbB-1/EGF-receptor but not to other ErbB proteins. *Oncogene* **12**, 1117-1125 (1996).

22. Levkowitz, G *et al.* Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol Cell* **4**, 1029-1040 (1999).

23. Torrisi, MR *et al.* Eps15 is recruited to the plasma membrane upon epidermal growth factor receptor activation and localizes to components of the endocytic pathway during receptor internalization. *Mol Biol Cell* **10**, 417-434 (1999).

24. Voisin, L *et al.* EGF receptor transactivation is obligatory for protein synthesis stimulation by G protein-coupled receptors. *Am J Physiol Cell Physiol* **283**, C446-C455 (2002).

25. Grant, S *et al.* Roles of ERBB family receptor tyrosine kinases, and downstream signaling pathways, in the control of cell growth and survival. *Front Biosci* **7**, d376-d389 (2002).

26. Verbeek, BS *et al.* Overexpression of EGFR and c-erbB2 causes enhanced cell migration in human breast cancer cells and NIH3T3 fibroblasts. *FEBS Lett* **425**, 145-150 (1998).

27. Lin, SY *et al.* Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat Cell Biol* **3**, 802-808 (2001).

28. Lo, HW *et al.* Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. *Cancer Cell* **7**, 575-589 (2005).

29. Pinkas-Kramarski, R *et al.* Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J* **15**, 2452-2467 (1996).

30. Riese, DJ *et al.* The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. *Mol Cell Biol* **15**, 5770-5776 (1995).

31. Gresik, EW *et al.* The EGF system in fetal development. *Eur J Morphol* **36 Suppl**, 92-97 (1998).

32. Miettinen, PJ *et al.* Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* **376**, 337-341 (1995).

33. Threadgill, DW *et al.* Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* **269**, 230-234 (1995).

34. Sibilia, M *et al.* Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* **269**, 234-238 (1995).

35. Lee, KF *et al.* Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394-398 (1995).

36. Gassmann, M *et al.* Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378**, 390-394 (1995).

37. Erickson, SL *et al.* ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. *Development* **124**, 4999-5011 (1997).

38. Bruns, CJ *et al.* Epidermal growth factor receptor blockade with C225 plus gemcitabine results in regression of human pancreatic carcinoma growing orthotopically in nude mice by antiangiogenic mechanisms. *Clin Cancer Res* **6**, 1936-1948 (2000).

39. Troyer, KL *et al.* Regulation of mouse mammary gland development and tumorigenesis by the ERBB signaling network. *J Mammary Gland Biol Neoplasia* **6**, 7-21 (2001).

40. Carpenter, G. Employment of the epidermal growth factor receptor in growth factor-independent signaling pathways. *J Cell Biol* **146**, 697-702 (1999).

41. Daub, H *et al.* Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* **379**, 557-560 (1996).

42. Thomas, SM *et al.* Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol* **13:513-609.**, 513-609 (1997).

43. Prenzel, N *et al.* EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**, 884-888 (1999).

44. Yamauchi, T *et al.* Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone. *Nature* **390**, 91-96 (1997).

45. Miyamoto, S *et al.* Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J Cell Biol* **135**, 1633-1642 (1996).

46. Jones, PL *et al.* Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the alpha v beta 3 integrin to promote epidermal growth factor receptor phosphorylation and growth. *J Cell Biol* **139**, 279-293 (1997).

47. Lin, RZ *et al.* Heat shock activates c-Src tyrosine kinases and phosphatidylinositol 3-kinase in NIH3T3 fibroblasts. *J Biol Chem* **272**, 31196-31202 (1997).

48. King, CR *et al.* Ligand-independent tyrosine phosphorylation of EGF receptor and the erbB-2/neu proto-oncogene product is induced by hyperosmotic shock. *Oncogene* **4**, 13-18 (1989).

49. Warmuth, I *et al.* Ultraviolet radiation induces phosphorylation of the epidermal growth factor receptor. *Cancer Res* **54**, 374-376 (1994).

50. Gilmore, AP *et al.* Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin-like growth factor receptor. *J Biol Chem* **277**, 27643-27650 (2002).

51. Dei Tos, AP *et al.* Assessing epidermal growth factor receptor expression in tumours: what is the value of current test methods? *Eur J Cancer* **41**, 1383-1392 (2005).

52. Nicholson, RI *et al.* EGFR and cancer prognosis. *Eur J Cancer* **37 Suppl 4**, S9-15 (2001).

53. Tangjitgamol, S *et al.* Expression of HER-2/neu, epidermal growth factor receptor, vascular endothelial growth factor, cyclooxygenase-2, estrogen receptor, and progesterone receptor in small cell and large cell neuroendocrine carcinoma of the uterine cervix: a clinicopathologic and prognostic study. *Int J Gynecol Cancer* **15**, 646-656 (2005).

54. Kim, GE *et al.* Synchronous coexpression of epidermal growth factor receptor and cyclooxygenase-2 in carcinomas of the uterine cervix: a potential predictor of poor survival. *Clin Cancer Res* **10**, 1366-1374 (2004).

55. Kersemaekers, AM *et al.* Oncogene alterations in carcinomas of the uterine cervix: overexpression of the epidermal growth factor receptor is associated with poor prognosis. *Clin Cancer Res* **5**, 577-586 (1999).

56. Kim, JW *et al.* Expression of epidermal growth factor receptor in carcinoma of the cervix. *Gynecol Oncol* **60**, 283-287 (1996).

57. Hale, RJ *et al.* Prognostic value of epidermal growth factor receptor expression in cervical carcinoma. *J Clin Pathol* **46**, 149-153 (1993).

58. Lee, CM *et al.* Expression of HER2neu (c-erbB-2) and epidermal growth factor receptor in cervical cancer: prognostic correlation with clinical characteristics, and comparison of manual and automated imaging analysis. *Gynecol Oncol* **93**, 209-214 (2004).

59. Kim, YT *et al.* Correlation between expression of EGFR and the prognosis of patients with cervical carcinoma. *Gynecol Oncol* **87**, 84-89 (2002).

60. Gibson, MK *et al.* Epidermal growth factor receptor, p53 mutation, and pathological response predict survival in patients with locally advanced esophageal cancer treated with preoperative chemoradiotherapy. *Clin Cancer Res* **9**, 6461-6468 (2003).

61. Itakura, Y *et al.* Epidermal growth factor receptor overexpression in esophageal carcinoma. An immunohistochemical study correlated with clinicopathologic findings and DNA amplification. *Cancer* **74**, 795-804 (1994).

62. Wilkinson, NW *et al.* Epidermal growth factor receptor expression correlates with histologic grade in resected esophageal adenocarcinoma. *J Gastrointest Surg* **8**, 448-453 (2004).

63. Gamboa-Dominguez, A *et al.* Epidermal growth factor receptor expression correlates with poor survival in gastric adenocarcinoma from Mexican patients: a multivariate analysis using a standardized immunohistochemical detection system. *Mod Pathol* **17**, 579-587 (2004).

64. Takehana, T *et al.* Expression of epidermal growth factor receptor in gastric carcinomas. *Clin Gastroenterol Hepatol* **1**, 438-445 (2003).

65. Yasui, W *et al.* Expression of epidermal growth factor receptor in human gastric and colonic carcinomas. *Cancer Res* **48**, 137-141 (1988).

66. Livasy, CA *et al.* EGFR expression and HER2/neu overexpression/amplification in endometrial carcinosarcoma. *Gynecol Oncol* **100**, 101-106 (2006).

67. Moinfar, F *et al.* Endometrial stromal sarcomas frequently express epidermal growth factor receptor (EGFR, HER-1): potential basis for a new therapeutic approach. *Am J Surg Pathol* **29**, 485-489 (2005).

68. Nyholm, HC *et al.* Expression of epidermal growth factor receptors in human endometrial carcinoma. *Int J Gynecol Pathol* **12**, 241-245 (1993).

69. Scambia, G *et al.* Significance of epidermal growth factor receptor expression in primary human endometrial cancer. *Int J Cancer* **56**, 26-30 (1994).

70. Hiwasa, T *et al.* Expression and localization of epidermal growth factor receptors and ras oncogene products in gynecologic tumors. *Eur J Gynaecol Oncol* **13**, 241-245 (1992).

71. Hanahan, D *et al.* The hallmarks of cancer. *Cell* **100**, 57-70 (2000).

72. Hirsch, FR *et al.* Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. *J Clin Oncol* **21**, 3798-3807 (2003).

73. Meert, AP *et al.* The role of EGF-R expression on patient survival in lung cancer: a systematic review with meta-analysis. *Eur Respir J* **20**, 975-981 (2002).

74. Umekita, Y *et al.* Co-expression of epidermal growth factor receptor and transforming growth factor-alpha predicts worse prognosis in breast-cancer patients. *Int J Cancer* **89**, 484-487 (2000).

75. Moscatello, DK *et al.* Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res* **55**, 5536-5539 (1995).

76. Libermann, TA *et al.* Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature* **313**, 144-147 (1985).

77. Garcia, dP, I *et al.* Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. *Cancer Res* **53**, 3217-3220 (1993).

78. Paez, JG *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304**, 1497-1500 (2004).

79. Lynch, TJ *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* **350**, 2129-2139 (2004).

80. Pao, W *et al.* EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A* **101**, 13306-13311 (2004).

81. Sordella, R *et al.* Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science* **305**, 1163-1167 (2004).

82. Barber, TD *et al.* Somatic mutations of EGFR in colorectal cancers and glioblastomas. *N Engl J Med* **351**, 2883 (2004).

83. Lee, JW *et al.* Somatic mutations of EGFR gene in squamous cell carcinoma of the head and neck. *Clin Cancer Res* **11**, 2879-2882 (2005).

84. Ozanne, B *et al.* Over-expression of the EGF receptor is a hallmark of squamous cell carcinomas. *J Pathol* **149**, 9-14 (1986).

85. Tang, CK *et al.* Epidermal growth factor receptor vIII enhances tumorigenicity in human breast cancer. *Cancer Res* **60**, 3081-3087 (2000).

86. Mendelsohn, J. Epidermal growth factor receptor inhibition by a monoclonal antibody as anticancer therapy. *Clin Cancer Res* **3**, 2703-2707 (1997).

87. Woodburn, JR. The epidermal growth factor receptor and its inhibition in cancer therapy. *Pharmacol Ther* **82**, 241-250 (1999).

88. Ciardiello, F *et al.* A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. *Clin Cancer Res* **7**, 2958-2970 (2001).

89. Mendelsohn, J *et al.* The EGF receptor family as targets for cancer therapy. *Oncogene* **19**, 6550-6565 (2000).

90. Mendelsohn, J. The epidermal growth factor receptor as a target for cancer therapy. *Endocr Relat Cancer* **8**, 3-9 (2001).

91. Sato, JD *et al.* Biological effects in vitro of monoclonal antibodies to human epidermal growth factor receptors. *Mol Biol Med* **1**, 511-529 (1983).

92. Kawamoto, T *et al.* Growth stimulation of A431 cells by epidermal growth factor: identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc Natl Acad Sci U S A* **80**, 1337-1341 (1983).

93. Gill, GN *et al.* Monoclonal anti-epidermal growth factor receptor antibodies which are inhibitors of epidermal growth factor binding and antagonists of epidermal growth factor binding and antagonists of epidermal growth factor-stimulated tyrosine protein kinase activity. *J Biol Chem* **259**, 7755-7760 (1984).

94. Goldstein, NI *et al.* Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. *Clin Cancer Res* **1**, 1311-1318 (1995).

95. Fan, Z *et al.* Antitumor effect of anti-epidermal growth factor receptor monoclonal antibodies plus cis-diamminedichloroplatinum on well established A431 cell xenografts. *Cancer Res* **53**, 4637-4642 (1993).

96. Baselga, J *et al.* Antitumor effects of doxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies. *J Natl Cancer Inst* **85**, 1327-1333 (1993).

97. Huang, SM *et al.* Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. *Cancer Res* **59**, 1935-1940 (1999).

98. Milas, L *et al.* In vivo enhancement of tumor radioresponse by C225 antiepidermal growth factor receptor antibody. *Clin Cancer Res* **6**, 701-708 (2000).

99. Yang, XD *et al.* Eradication of established tumors by a fully human monoclonal antibody to the epidermal growth factor receptor without concomitant chemotherapy. *Cancer Res* **59**, 1236-1243 (1999).

100. Wikstrand, CJ *et al.* Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. *Cancer Res* **55**, 3140-3148 (1995).

101. Kuan, CT *et al.* EGF mutant receptor vIII as a molecular target in cancer therapy. *Endocr Relat Cancer* **8**, 83-96 (2001).

102. Sampson, JH *et al.* Subcutaneous vaccination with irradiated, cytokine-producing tumor cells stimulates CD8+ cell-mediated immunity against tumors located in the "immunologically privileged" central nervous system. *Proc Natl Acad Sci U S A* **93**, 10399-10404 (1996).

103. Sampson, JH *et al.* Unarmed, tumor-specific monoclonal antibody effectively treats brain tumors. *Proc Natl Acad Sci U S A* **97**, 7503-7508 (2000).

104. Masui, H *et al.* Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. *Cancer Res* **44**, 1002-1007 (1984).

105. Markowitz, SD *et al.* Growth stimulation by coexpression of transforming growth factor-alpha and epidermal growth factor-receptor in normal and adenomatous human colon epithelium. *J Clin Invest* **86**, 356-362 (1990).

106. Kawamoto, T *et al.* Relation of epidermal growth factor receptor concentration to growth of human epidermoid carcinoma A431 cells. *J Biol Chem* **259**, 7761-7766 (1984).

107. Wu, X *et al.* Apoptosis induced by an anti-epidermal growth factor receptor monoclonal antibody in a human colorectal carcinoma cell line and its delay by insulin. *J Clin Invest* **95**, 1897-1905 (1995).

108. Peng, D *et al.* Anti-epidermal growth factor receptor monoclonal antibody 225 up-regulates p27KIP1 and induces G1 arrest in prostatic cancer cell line DU145. *Cancer Res* **56**, 3666-3669 (1996).

109. Wu, X *et al.* Involvement of p27KIP1 in G1 arrest mediated by an anti-epidermal growth factor receptor monoclonal antibody. *Oncogene* **12**, 1397-1403 (1996).

110. Fan, Z *et al.* Reciprocal changes in p27(Kip1) and p21(Cip1) in growth inhibition mediated by blockade or overstimulation of epidermal growth factor receptors. *Clin Cancer Res* **3**, 1943-1948 (1997).

111. Perrotte, P *et al.* Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res* **5**, 257-265 (1999).

112. Albanell, J *et al.* Activated extracellular signal-regulated kinases: association with epidermal growth factor receptor/transforming growth factor alpha expression in head and neck squamous carcinoma and inhibition by anti-epidermal growth factor receptor treatments. *Cancer Res* **61**, 6500-6510 (2001).

113. Janmaat, ML *et al.* Response to Epidermal Growth Factor Receptor Inhibitors in Non-Small Cell Lung Cancer Cells: Limited Antiproliferative Effects and Absence of Apoptosis Associated with Persistent Activity of Extracellular Signal-regulated Kinase or Akt Kinase Pathways. *Clin Cancer Res* **9**, 2316-2326 (2003).

114. Liu, B *et al.* Induction of apoptosis and activation of the caspase cascade by anti-EGF receptor monoclonal antibodies in DiFi human colon cancer cells do not involve the c-jun N-terminal kinase activity. *Br J Cancer* **82**, 1991-1999 (2000).

115. Tortora, G *et al.* Cooperative inhibitory effect of novel mixed backbone oligonucleotide targeting protein kinase A in combination with docetaxel and anti-epidermal growth factor-receptor antibody on human breast cancer cell growth. *Clin Cancer Res* **5**, 875-881 (1999).

116. Mandal, M *et al.* Nuclear targeting of Bax during apoptosis in human colorectal cancer cells. *Oncogene* **17**, 999-1007 (1998).

117. Inoue, K *et al.* Paclitaxel enhances the effects of the anti-epidermal growth factor receptor monoclonal antibody ImClone C225 in mice with metastatic human bladder transitional cell carcinoma. *Clin Cancer Res* **6**, 4874-4884 (2000).

118. Ciardiello, F *et al.* Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. *Clin Cancer Res* **5**, 909-916 (1999).

119. Huang, SM *et al.* Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. *Clin Cancer Res* **6**, 2166-2174 (2000).

120. Baumann, M *et al.* Targeting the epidermal growth factor receptor in radiotherapy: radiobiological mechanisms, preclinical and clinical results. *Radiother Oncol* **72**, 257-266 (2004).

121. Sepp-Lorenzino, L *et al. Proc Am Assoc Cancer Res* **37**, 421 (1996).

122. Ciardiello, F *et al.* Antitumor activity of combined blockade of epidermal growth factor receptor and protein kinase A. *J Natl Cancer Inst* **88**, 1770-1776 (1996).

123. Petit, AM *et al.* Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* **151**, 1523-1530 (1997).

124. Cunningham, D *et al.* Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* **351**, 337-345 (2004).

125. Bonner, JA *et al.* Cetuximab prolongs survival in patients with locoregionally advanced squamous cell carcinoma of head and neck: A phase III study of high dose radiation therapy with or without cetuximab. *Proc Am Soc Clin Oncol* **23**, A5507 (2004).

126. Honegger, AM *et al.* Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. *Cell* **51**, 199-209 (1987).

127. Chen, WS *et al.* Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. *Nature* **328**, 820-823 (1987).

128. Noonberg, SB *et al.* Tyrosine kinase inhibitors targeted to the epidermal growth factor receptor subfamily: role as anticancer agents. *Drugs* **59**, 753-767 (2000).

129. Arteaga, CL *et al.* Tyrosine kinase inhibitors-ZD1839 (Iressa). *Curr Opin Oncol* **13**, 491-498 (2001).

130. Ciardiello, F *et al.* Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* **6**, 2053-2063 (2000).

131. Sirotnak, FM *et al.* Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* **6**, 4885-4892 (2000).

132. Moulder, SL *et al.* Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells in vitro and in vivo. *Cancer Res* **61**, 8887-8895 (2001).

133. Magne, N *et al.* Influence of epidermal growth factor receptor (EGFR), p53 and intrinsic MAP kinase pathway status of tumour cells on the antiproliferative effect of ZD1839 ("Iressa"). *Br J Cancer* **86**, 1518-1523 (2002).

134. Moasser, MM *et al.* The tyrosine kinase inhibitor ZD1839 ("Iressa") inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. *Cancer Res* **61**, 7184-7188 (2001).

135. Anderson, NG *et al.* ZD1839 (Iressa), a novel epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, potently inhibits the growth of EGFR-positive cancer cell lines with or without erbB2 overexpression. *Int J Cancer* **94**, 774-782 (2001).

136. Arteaga, CL. Epidermal growth factor receptor dependence in human tumors: more than just expression? *Oncologist* **7 Suppl 4**, 31-39 (2002).

137. Wakeling, AE *et al.* ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. *Cancer Res* **62**, 5749-5754 (2002).

138. Arteaga, CL *et al.* Unliganded epidermal growth factor receptor dimerization induced by direct interaction of quinazolines with the ATP binding site. *J Biol Chem* **272**, 23247-23254 (1997).

139. Lichtner, RB *et al.* Signaling-inactive epidermal growth factor receptor/ligand complexes in intact carcinoma cells by quinazoline tyrosine kinase inhibitors. *Cancer Res* **61**, 5790-5795 (2001).

140. Engelman, JA *et al.* ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. *Proc Natl Acad Sci U S A* **102**, 3788-3793 (2005).

141. Busse, D *et al.* Reversible G(1) arrest induced by inhibition of the epidermal growth factor receptor tyrosine kinase requires up-regulation of p27(KIP1) independent of MAPK activity. *J Biol Chem* **275**, 6987-6995 (2000).

142. She, QB *et al.* Resistance to gefitinib in PTEN-null HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacologic modulation of constitutive phosphatidylinositol 3'-kinase/Akt pathway signaling. *Clin Cancer Res* **9**, 4340-4346 (2003).

143. Bianco, R *et al.* Loss of PTEN/MMAC1/TEP in EGF receptor-expressing tumor cells counteracts the antitumor action of EGFR tyrosine kinase inhibitors. *Oncogene* **22**, 2812-2822 (2003).

144. Li, B *et al.* Resistance to small molecule inhibitors of epidermal growth factor receptor in malignant gliomas. *Cancer Res* **63**, 7443-7450 (2003).

145. Samuels, Y *et al.* High frequency of mutations of the PIK3CA gene in human cancers. *Science* **304**, 554 (2004).

146. Bos, JL. ras oncogenes in human cancer: a review. *Cancer Res* **49**, 4682-4689 (1989).

147. Stambolic, V *et al.* Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95**, 29-39 (1998).

148. Janmaat, ML *et al.* Enhanced cytotoxicity induced by gefitinib and specific inhibitors of the Ras or phosphatidyl inositol-3 kinase pathways in non-small cell lung cancer cells. *Int J Cancer* **118**, 209-214 (2006).

149. Pao, W *et al.* KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med* **2**, e17 (2005).

150. Jones, HE *et al.* Insulin-like growth factor-I receptor signalling and acquired resistance to gefitinib (ZD1839; Iressa) in human breast and prostate cancer cells. *Endocr Relat Cancer* **11**, 793-814 (2004).

151. Magne, N *et al.* Sequence-dependent effects of ZD1839 ('Iressa') in combination with cytotoxic treatment in human head and neck cancer. *Br J Cancer* **86**, 819-827 (2002).

152. Williams, KJ *et al.* ZD1839 ('Iressa'), a specific oral epidermal growth factor receptor-tyrosine kinase inhibitor, potentiates radiotherapy in a human colorectal cancer xenograft model. *Br J Cancer* **86**, 1157-1161 (2002).

153. Huang, SM *et al.* Modulation of radiation response and tumor-induced angiogenesis after epidermal growth factor receptor inhibition by ZD1839 (Iressa). *Cancer Res* **62**, 4300-4306 (2002).

154. Elkind, NB *et al.* Multidrug transporter ABCG2 prevents tumor cell death induced by the epidermal growth factor receptor inhibitor Iressa (ZD1839, Gefitinib). *Cancer Res* **65**, 1770-1777 (2005).

155. Nakamura, Y *et al.* Gefitinib ("Iressa", ZD1839), an epidermal growth factor receptor tyrosine kinase inhibitor, reverses breast cancer resistance protein/ABCG2-mediated drug resistance. *Cancer Res* **65**, 1541-1546 (2005).

156. Yanase, K *et al.* Gefitinib reverses breast cancer resistance protein-mediated drug resistance. *Mol Cancer Ther* **3**, 1119-1125 (2004).

157. Stewart, CF *et al.* Gefitinib enhances the antitumor activity and oral bioavailability of irinotecan in mice. *Cancer Res* **64**, 7491-7499 (2004).

158. Naruse, I *et al.* Antitumor activity of the selective epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) Iressa (ZD1839) in an EGFR-expressing multidrug-resistant cell line in vitro and in vivo. *Int J Cancer* **98**, 310-315 (2002).

159. Normanno, N *et al.* Cooperative inhibitory effect of ZD1839 (Iressa) in combination with trastuzumab (Herceptin) on human breast cancer cell growth. *Ann Oncol* **13**, 65-72 (2002).

160. Moulder, SL *et al.* A Phase I/II Trial of Trastuzumab and Gefitinib in Patients with Metastatic Breast Cancer That Overexpresses HER2/neu (ErbB-2). *Clin Breast Cancer* **4**, 142-145 (2003).

161. Ciardiello, F *et al.* Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res* **7**, 1459-1465 (2001).

162. Hirata, A *et al.* ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase. *Cancer Res* **62**, 2554-2560 (2002).

163. Mandal, M *et al.* Inhibition of p21-activated kinase 1, directional cell motility and invasion of growth-factor-activated human cancer cells by the selective epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) ZD1839 (´Iressa´). *Proc Am Assoc Cancer Res* **43**, A786 (2002).

164. Moyer, JD *et al.* Induction of apoptosis and cell cycle arrest by CP-358,774, an inhibitor of epidermal growth factor receptor tyrosine kinase. *Cancer Res* **57**, 4838-4848 (1997).

165. Pollack, VA *et al.* Inhibition of epidermal growth factor receptor-associated tyrosine phosphorylation in human carcinomas with CP-358,774: dynamics of receptor inhibition in situ and antitumor effects in athymic mice. *J Pharmacol Exp Ther* **291**, 739-748 (1999).

166. Ng, SS *et al.* Effects of the epidermal growth factor receptor inhibitor OSI-774, Tarceva, on downstream signaling pathways and apoptosis in human pancreatic adenocarcinoma. *Mol Cancer Ther* **1**, 777-783 (2002).

167. Tan, AR *et al.* Evaluation of biologic end points and pharmacokinetics in patients with metastatic breast cancer after treatment with erlotinib, an epidermal growth factor receptor tyrosine kinase inhibitor. *J Clin Oncol* **22**, 3080-3090 (2004).

168. Malik, SN *et al.* Pharmacodynamic evaluation of the epidermal growth factor receptor inhibitor OSI-774 in human epidermis of cancer patients. *Clin Cancer Res* **9**, 2478-2486 (2003).

169. Akita, RW *et al.* Preclinical studies with Erlotinib (Tarceva). *Semin Oncol* **30**, 15-24 (2003).

170. Lal, A *et al.* Mutant epidermal growth factor receptor up-regulates molecular effectors of tumor invasion. *Cancer Res* **62**, 3335-3339 (2002).

171. Gieseg, MA *et al.* Evidence for epidermal growth factor receptor-enhanced chemosensitivity in combinations of cisplatin and the new irreversible tyrosine kinase inhibitor CI-1033. *Anticancer Drugs* **12**, 683-690 (2001).

172. Erlichman, C *et al.* The HER tyrosine kinase inhibitor CI1033 enhances cytotoxicity of 7-ethyl-10-hydroxycamptothecin and topotecan by inhibiting breast cancer resistance protein-mediated drug efflux. *Cancer Res* **61**, 739-748 (2001).

173. Rao, GS *et al.* Radiosensitization of human breast cancer cells by a novel ErbB family receptor tyrosine kinase inhibitor. *Int J Radiat Oncol Biol Phys* **48**, 1519-1528 (2000).

174. Nelson, JM *et al.* Akt, MAPK (Erk1/2), and p38 act in concert to promote apoptosis in response to ErbB receptor family inhibition. *J Biol Chem* **276**, 14842-14847 (2001).

175. Motoyama, AB *et al.* The efficacy of ErbB receptor-targeted anticancer therapeutics is influenced by the availability of epidermal growth factor-related peptides. *Cancer Res* **62**, 3151-3158 (2002).

176. Mellinghoff, IK *et al.* Growth inhibitory effects of the dual ErbB1/ErbB2 tyrosine kinase inhibitor PKI-166 on human prostate cancer xenografts. *Cancer Res* **62**, 5254-5259 (2002).

177. Baker, CH *et al.* Blockade of vascular endothelial growth factor receptor and epidermal growth factor receptor signaling for therapy of metastatic human pancreatic cancer. *Cancer Res* **62**, 1996-2003 (2002).

178. Brandt, R *et al.* Mammary glands reconstituted with Neu/ErbB2 transformed HC11 cells provide a novel orthotopic tumor model for testing anti-cancer agents. *Oncogene* **20**, 5459-5465 (2001).

179. Solorzano, CC *et al.* Optimization for the blockade of epidermal growth factor receptor signaling for therapy of human pancreatic carcinoma. *Clin Cancer Res* **7**, 2563-2572 (2001).

180. Bruns, CJ *et al.* Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res* **60**, 2926-2935 (2000).

181. Xia, W *et al.* Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* **21**, 6255-6263 (2002).

182. Mullin, RJ *et al.* Combination therapy with the dual EGFR-ERBB-2 tyrosine kinase inhibitor GW572016. *Proc Am Soc Clin Oncol* **22**, A970 (2003).

183. Wissner, A *et al.* Syntheses and EGFR and HER-2 kinase inhibitory activities of 4-anilinoquinoline-3-carbonitriles: analogues of three important 4-anilinoquinazolines currently undergoing clinical evaluation as therapeutic antitumor agents. *Bioorg Med Chem Lett* **12**, 2893-2897 (2002).

184. Natarajan, M *et al.* EKB-569 attenuates radiation triggered survival response in squamous cell carcinoma. *Proc Am Soc Clin Oncol* **22**, A993 (2003).

185. Hamann, MT *et al.* Kahalalide F: a bioactive depsipeptide from the sacoglossan mollusk *Elysia rufescens* and the green alga *Bryopsis* sp. *J Am Chem Soc* **115**, 5825-5826 (1993).

186. Hamann, MT *et al.* Kahalalides: Bioactive Peptides from a Marine Mollusk Elysia rufescens and Its Algal Diet Bryopsis sp.(1). *J Org Chem* **61**, 6594-6600 (1996).

187. Wosikowski, K *et al.* Identification of epidermal growth factor receptor and c-erbB2 pathway inhibitors by correlation with gene expression patterns. *J Natl Cancer Inst* **89**, 1505-1515 (1997).

188. Faircloth, GT *et al.* Selective antitumor activity of Kahalalide F, a marine-derived cyclic depsipeptide. *Proc Am Assoc Cancer Res* **42**, 213 (2001).

189. Suarez, Y *et al.* Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. *Mol Cancer Ther* **2**, 863-872 (2003).

190. Garcia-Rocha, M *et al.* The antitumoral compound Kahalalide F acts on cell lysosomes. *Cancer Lett* **99**, 43-50 (1996).

191. Janmaat, ML *et al.* Kahalalide F induces necrosis-like cell death that involves depletion of ErbB3 and inhibition of Akt signaling. *Mol Pharmacol* **68**, 502-510 (2005).

192. Jimeno, J *et al.* Progress in the clinical development of new marine-derived anticancer compounds. *Anticancer Drugs* **15**, 321-329 (2004).

193. Faircloth, GT *et al.* Preclinical development of Kahalalide F, a new marine compound selected for clinical studies. *Proc Am Assoc Cancer Res* **41**, 600 (2000).

194. Medina, LA *et al.* Investigation of the effects of Kahalalide F (PM92102) against tumor specimens taken directly from patients. *Proc Am Assoc Cancer Res* **42**, 213 (2001).

195. Shao, L *et al. In vitro* anti-proliferative effect on sarcoma cells of ET-743 and other marine chemotherapeutics. *Proc Am Assoc Cancer Res* **42**, 203 (2001).

196. Gomez, SG *et al.* In vitro toxicity of three new antitumoral drugs (trabectedin, aplidin, and kahalalide F) on hematopoietic progenitors and stem cells. *Exp Hematol* **31**, 1104-1111 (2003).

197. Ciruelos, C *et al.* A phase I clinical and pharmokinetic (PK) study with Kahalalide F (KF) in patients (pts) with advanced solid tumors (AST) with a continuous weekly (W) 1-hour iv infusion schedule. *Eur J Cancer* **38 Suppl 7**, S33 (2002).

198. Herbst, RS *et al.* Selective Oral Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor ZD1839 Is Generally Well-Tolerated and Has Activity in Non-Small-Cell Lung Cancer and Other Solid Tumors: Results of a Phase I Trial. *J Clin Oncol* **20**, 3815-3825 (2002).

199. Baselga, J *et al.* Phase I safety, pharmacokinetic, and pharmacodynamic trial of ZD1839, a selective oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with five selected solid tumor types. *J Clin Oncol* **20**, 4292-4302 (2002).

200. Ranson, M *et al.* ZD1839, a selective oral epidermal growth factor receptor-tyrosine kinase inhibitor, is well tolerated and active in patients with solid, malignant tumors: results of a phase I trial. *J Clin Oncol* **20**, 2240-2250 (2002).

201. Albanell, J *et al.* Pharmacodynamic studies of the epidermal growth factor receptor inhibitor ZD1839 in skin from cancer patients: histopathologic and molecular consequences of receptor inhibition. *J Clin Oncol* **20**, 110-124 (2002).

202. Negoro, S *et al.* Final Results of a Phase I Intermittent Dose-Escalation Trial of ZD1839 ('Iressa') In Japanese Patients With Various Solid Tumours. *Proc Am Soc Clin Oncol* **20**, A1292 (2001).

203. Fukuoka M *et al.* Final results from a phase II trial of ZD1839 ('Iressa') for patients with advanced non-small-cell lung cancer (IDEAL 1). *Proc Am Soc Clin Oncol* **21**, A1188 (2002).

204. Kris, MG *et al.* Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA* **290**, 2149-2158 (2003).

205. Cohen, EE *et al.* Phase II trial of ZD1839 in recurrent or metastatic squamous cell carcinoma of the head and neck. *J Clin Oncol* **21**, 1980-1987 (2003).

206. Baselga, J *et al.* Phase II and tumor pharmacodynamic study of gefitinib in patients with advanced breast cancer. *J Clin Oncol* **23**, 5323-5333 (2005).

207. Giaccone, G *et al.* Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 1. *J Clin Oncol* **22**, 777-784 (2004).

208. Herbst, RS *et al.* Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 2. *J Clin Oncol* **22**, 785-794 (2004).

209. Thatcher, N *et al.* Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). *Lancet* **366**, 1527-1537 (2005).

210. Hidalgo, M *et al.* Phase I and pharmacologic study of OSI-774, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies. *J Clin Oncol* **19**, 3267-3279 (2001).

211. Ratain, MJ *et al.* Phase I trial of erlotinib (OSI-774) in combination with gemcitabine (G) and cisplatin (P) in patients with advanced solid tumors. *Proc Am Soc Clin Oncol* **21** (2002).

212. Perez-Soler, R *et al.* A phase II trial of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor OSI-774, following platinum-based chemotherapy in patients (pts) with advanced, EGFR-expressing, non-small cell lung cancer (NSCLC). *Proc Am Soc Clin Oncol* **20**, A1235 (2001).

213. Perez-Soler, R. Phase II clinical trial data with the epidermal growth factor receptor tyrosine kinase inhibitor erlotinib (OSI-774) in non-small-cell lung cancer. *Clin Lung Cancer* **6 Suppl 1**, S20-S23 (2004).

214. Gordon, AN *et al.* Efficacy and safety of erlotinib HCl, an epidermal growth factor receptor (HER1/EGFR) tyrosine kinase inhibitor, in patients with advanced ovarian carcinoma: results from a phase II multicenter study. *Int J Gynecol Cancer* **15**, 785-792 (2005).

215. Soulieres, D *et al.* Multicenter phase II study of erlotinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with recurrent or metastatic squamous cell cancer of the head and neck. *J Clin Oncol* **22**, 77-85 (2004).

216. Philip, PA *et al.* Phase II study of Erlotinib (OSI-774) in patients with advanced hepatocellular cancer. *J Clin Oncol* **23**, 6657-6663 (2005).

217. Senzer, NN *et al.* Phase 2 Evaluation of OSI-774, a Potent Oral Antagonist of the EGFR-TK in Patients with Advanced Squamous Cell Carcinoma of the Head and Neck. *Proc Am Soc Clin Oncol* **20**, A6 (2001).

218. Bulgaru, AM *et al.* Erlotinib (Tarceva): a promising drug targeting epidermal growth factor receptor tyrosine kinase. *Expert Rev Anticancer Ther* **3**, 269-279 (2003).

219. Forero, L *et al.* Phase I, pharmacokinetic (PK) and biologic study of OSI-774, a selective epidermal growth factor receptor (EGFR) tyrosine kinase (TK) inhibitor in combination with paclitaxel and carboplatin. *Proc Am Soc Clin Oncol* **21**, A1908 (2002).

220. Forouzesh, B *et al.* Phase I, pharmacokinetic (PK), and biological studies of the epidermal growth factor-tyrosine kinase (EGFR-TK) inhibitor OSI-774 in combination with docetaxel. *Proc Am Soc Clin Oncol* **21**, A81 (2002).

221. Gatzemeier, U *et al.* Results of a phase III trial of erlotinib (OSI-774) combined with cisplatin and gemcitabine (GC) chemotherapy in advanced non-small cell lung cancer (NSCLC). *Proc Am Soc Clin Oncol* **23** (2004).

222. Herbst, RS *et al.* TRIBUTE: a phase III trial of erlotinib hydrochloride (OSI-774) combined with carboplatin and paclitaxel chemotherapy in advanced non-small-cell lung cancer. *J Clin Oncol* **23**, 5892-5899 (2005).

223. Shepherd, FA *et al.* Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* **353**, 123-132 (2005).

224. Moore, MJ *et al.* Erlotinib plus gemcitabine compared to gemcitabine alone in patients with advanced pancreatic cancer. A phase III trial of the National Cancer Institute of Canada Clinical Trials Group [NCIC-CTG]. *Proc Am Soc Clin Oncol* **24** (2005).

225. Bell, DW *et al.* Epidermal growth factor receptor mutations and gene amplification in non-small-cell lung cancer: molecular analysis of the IDEAL/INTACT gefitinib trials. *J Clin Oncol* **23**, 8081-8092 (2005).

226. Cappuzzo, F *et al.* Gefitinib in Pretreated Non-Small-Cell Lung Cancer (NSCLC): Analysis of Efficacy and Correlation With HER2 and Epidermal Growth Factor Receptor Expression in Locally Advanced or Metastatic NSCLC. *J Clin Oncol* **21**, 2658-2663 (2003).

227. Fukuoka, M *et al.* Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. *J Clin Oncol* **21**, 2237-2246 (2003).

228. Cappuzzo, F *et al.* Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. *J Natl Cancer Inst* **97**, 643-655 (2005).

229. Eberhard, DA *et al.* Mutations in the epidermal growth factor receptor and in KRAS are predictive and prognostic indicators in patients with non-small-cell lung cancer treated with chemotherapy alone and in combination with erlotinib. *J Clin Oncol* **23**, 5900-5909 (2005).

230. Tsao, MS *et al.* Erlotinib in lung cancer - molecular and clinical predictors of outcome. *N Engl J Med* **353**, 133-144 (2005).

231. Clark, GM *et al.* Rash severity is predictive of increased survival with erlotinib HCl. *Proc Am Soc Clin Oncol* **22**, A786 (2003).

232. Pao, W *et al.* Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* **2**, e73 (2005).

233. Kobayashi, S *et al.* EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* **352**, 786-792 (2005).

234. Toyooka, S *et al.* EGFR mutation and response of lung cancer to gefitinib. *N Engl J Med* **352**, 2136 (2005).

235. Shih, JY *et al.* EGFR mutation conferring primary resistance to gefitinib in non-small-cell lung cancer. *N Engl J Med* **353**, 207-208 (2005).

236. Kobayashi, S *et al.* An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor. *Cancer Res* **65**, 7096-7101 (2005).

237. Carter, TA *et al.* Inhibition of drug-resistant mutants of ABL, KIT, and EGF receptor kinases. *Proc Natl Acad Sci U S A* **102**, 11011-11016 (2005).

238. Zinner RG *et al.* A phase I clinical and biomarker study of the novel pan-erB tyrosine kinase inhibitor CI-1033, in patients with solid tumors. *Clin Cancer Res* **7**, 566a (2001).

239. Nemunaitis J. *et al.* A phase 1 trial of CI-1033, a pan-erbB tyrosine kinase inhibitor, given daily for 14 days every 3 weeks, in patients with advanced solid tumors. *Proc Am Soc Clin Oncol* **22**, A974 (2003).

240. Hoekstra, R *et al.* Phase I and pharmacologic study of PKI166, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies. *Clin Cancer Res* **11**, 6908-6915 (2005).

241. Belanger, M *et al.* A phase II, open-label, multicenter study of GW572016 in patients with metastatic colorectal cancer refractory to 5-FU5-FU in combination with irinotecan and/or oxaliplatin. *Proc Am Soc Clin Oncol* **22**, A978 (2003).

242. Kaplan, EH *et al.* A phase II, open-label, multicenter study of GW572016 in patients with trastuzumab refractory metastatic breast cancer. *Proc Am Soc Clin Oncol* **22**, A981 (2003).

243. DeBono, JS *et al.* Phase I and pharmacokinetic (PK) study of oral GW572016, a potent reversible dual inhibitor of both erbB1 and erbB2 tyrosine kinase (TK), administered in combination with capecitabine. *Proc Am Soc Clin Oncol* **22**, A901 (2003).

244. Burris, HA, III *et al.* Phase I safety, pharmacokinetics, and clinical activity study of lapatinib (GW572016), a reversible dual inhibitor of epidermal growth factor receptor tyrosine kinases, in heavily pretreated patients with metastatic carcinomas. *J Clin Oncol* **23**, 5305-5313 (2005).

245. Spector, NL *et al.* Study of the biologic effects of lapatinib, a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, on tumor growth and survival pathways in patients with advanced malignancies. *J Clin Oncol* **23**, 2502-2512 (2005).

246. Bence, AK *et al.* Phase I pharmacokinetic studies evaluating single and multiple doses of oral GW572016, a dual EGFR-ErbB2 inhibitor, in healthy subjects. *Invest New Drugs* **23**, 39-49 (2005).

247. DeSimone, PA *et al.* A phase I study to investigate the safety, tolerability, and pharmacokinetics of single oral escalating doses of GW572016 in healthy volunteers. *Proc Am Soc Clin Oncol* **22**, A275 (2003).

248. Burris, HA *et al.* A phase I study of GW572016 in patients with solid tumors. *Proc Am Soc Clin Oncol* **22**, A994 (2003).

249. Hidalgo, M *et al.* Phase I trial of EKB-569, an irreversible inhibitor of the epidermal growth factor receptor (EGFR), in patients with advanced solid tumors. *Proc Am Soc Clin Oncol* **21**, A65 (2002).

250. Morgan, JA *et al.* Preliminary report of a phase I study of EKB-569, an irreversible inhibitor of the epidermal growth factor receptor (EGFR), given in combination with gemcitabine to patients with advanced pancreatic cancer. *Proc Am Soc Clin Oncol* **22**, A788 (2003).

251. Salazar, R *et al.* A phase 1/2A open-label study of EKB-569 in combination with CPT-11/5-FU/LV (FOLFIRI) in patients with advanced colorectal cancer. *Proc Am Soc Clin Oncol* **22**, A888 (2003).

Chapter 2

Response to epidermal growth factor receptor inhibitors in non-small-cell lung cancer cells: limited antiproliferative effects and absence of apoptosis, associated with persistent activity of Erk or Akt kinase pathways.

Maarten L Janmaat
Frank AE Kruyt
José A Rodriguez
Giuseppe Giaccone

Clinical Cancer Research 2003, 9: 2316-2326.

Abstract

The epidermal growth factor receptor (EGFR) is an important novel target for anticancer therapy. In this study, we examined the molecular mechanisms that underlie the antitumor effects of the anti-EGFR monoclonal antibody C225 (Cetuximab) and the selective EGFR-tyrosine kinase inhibitor ZD1839 ('Iressa') in non-small cell lung cancer (NSCLC) cell lines. Cell growth, assessed by the MTT assay, was inhibited at low concentrations of ZD1839 and C225 in control A431 cells, whereas the NSCLC cell lines were comparatively more resistant. In A431 cells, but not in the NSCLC cells, ZD1839 treatment resulted in a modest increase in DNA fragmentation, the externalisation of phosphatidyl-serine, and the activation of caspase-3, known markers of apoptotic cell death. However, PARP-cleavage was not detected and caspase inhibition by zVAD-fmk partially reduced ZD1839-generated DNA fragmentation. Overexpression of the anti-apoptotic protein Bcl-2 in A431 cells suppressed the cytotoxity upon anti-EGFR treatment. These results thus demonstrate that the toxic effect of ZD1839 in A431 cells is caused by a form of cell death that involves a mitochondrial step and is at least in part dependent on caspase activation. EGFR expression levels showed no significant correlation with sensitivity to ZD1839 and C225. Evaluation of the MEK/Erk and PI3K/Akt pathways showed considerable inhibition of these pathways by ZD1839 and C225 in A431 cells, whereas one or both of these pathways remained active upon anti-EGFR treatment in NSCLC cells. In addition, treatment with specific inhibitors of MEK or PI3K resulted in a smaller effect on proliferation than simultaneous treatment with both inhibitors, while induction of apoptosis was observed only when both pathways were blocked. Together, these data suggest that persistent activity of either of these signalling pathways is involved in the lack of sensitivity of NSCLC cell lines to EGFR inhibitors.

Introduction

High expression of the EGFR[2] and/or its ligands is common in several tumor types, including head and neck cancer, breast cancer, ovary cancer, and NSCLC, and correlates with more aggressive disease, resistance to chemotherapy, and a poor prognosis [1]. Moreover, in lung and other tumors coexpression of EGFR and its ligand TGFα is common, suggesting an important role for the EGFR/TGFα autocrine loop in cancer [1,2].

EGFR is a 170 kD transmembrane protein consisting of an extracellular ligand binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. Upon ligand binding, the receptor dimerizes, either as homodimer or as heterodimer with other members of the ErbB family of receptor tyrosine kinases, preferably ErbB2 (HER2), and undergoes autophosphorylation at specific tyrosine residues of the intracellular domain. The phosphorylated tyrosine residues then serve as docking sites for proteins like Grb2, Shc and PLC that, in turn, activate downstream signalling pathways, including the Ras/MEK/Erk and the PI3K/Akt pathway, that regulate transcription factors and other proteins involved in biological responses as proliferation, cell motility, angiogenesis, cell survival, and differentiation [3,4].

Two main strategies have been developed to target the EGFR and block its activation in cancer cells [5,6]. Monoclonal antibodies against the extracellular domain of EGFR compete with ligand for receptor binding, thereby preventing kinase activation. An example of this is the human-mouse chimeric Mab C225 that has a high affinity for EGFR and is currently in phase II and III clinical trials in head and neck cancer, colorectal cancer and other tumor types [7]. Other promising anti-EGFR agents are EGFR-TKIs that prevent autophosphorylation of EGFR by physical interaction with its intracellular kinase domain. ZD1839 ('Iressa') is an orally active, selective EGFR-TKI [8] that is currently in phase II-III clinical trials, in patients with NSCLC, among several tumor types [9-11].

Various preclinical studies have demonstrated antitumor effects of C225 and ZD1839 in a variety of cell types and mouse xenografts as single agents and in combination with other anticancer therapies, in particular chemotherapeutic agents and radiation [12-15]. As single agents, the EGFR antagonists induce *in vitro* growth inhibition and, in some cell lines, apoptosis [16,17].

To define potential markers that could predict the outcome of anti-EGFR treatment, we investigated the molecular mechanisms that underlie the antitumor effects of the EGFR antagonists C225 and ZD1839 in NSCLC cells. We used a panel of four NSCLC cell lines and the highly EGFR expressing A431 cell line and determined *in vitro* cytotoxic and cytostatic effects after exposure to ZD1839 or C225. The EGFR inhibitors induced effective growth inhibition of A431 cells, while all of the NSCLC cell lines were more resistant. Cytotoxic effects were only observed in the A431 cell line, in which the role of apoptosis was further investigated. Protein expression levels of neither EGFR nor ErbB2 correlated with sensitivity to EGFR antagonists. In addition, the activity of kinase pathways downstream of the EGFR via MEK/Erk and PI3K/Akt was determined after treatment with EGFR inhibitors, showing persistent activity of at least one of these pathways in the NSCLC cell lines. Treatment with a combination of specific chemical inhibitors targeting MEK and PI3K resulted in the induction of apoptosis and effective inhibition of cell growth. Together, the results indicate that persistent activity of the MEK/Erk and PI3K/Akt kinase pathways can contribute to resistance of NSCLC cells to EGFR inhibitors.

Materials and Methods

Chemicals

Anti-EGFR Mab C225 was kindly provided by Merck, Germany, at a concentration of 2.0 mg/ml. Kinase inhibitors were provided as pure substances and diluted in DMSO. ZD1839 was a kind gift from AstraZeneca. U0126 and LY294002 were purchased from Cell Signalling Technology (Beverly, MA). The broad caspase inhibitor Carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk) was purchased from Enzyme Systems (Livermore, CA). The stock solution of cisplatin (Bristol-Meyers Squibb, Woerden, The Netherlands) was made in PBS.

Cells and culture conditions

RPMI 1640 (containing 2 mM L-glutamine) and DMEM were used as culture media and were supplemented with 10 % heat-inactivated fetal calf serum (Gibco BRL, Life technology, Breda, The Netherlands), 50 iu/ml penicillin and 50 µg/ml streptomycin. The human NSCLC cell lines NCI-H460 (H460), NCI-H1703 (H1703), and A549 cells were cultured in RPMI 1640, the human NSCLC cell line SW1573 and the vulval squamous cell carcinoma cell line A431 were cultured in DMEM. Cells were grown at 37° C in a humidified atmosphere with 5 % CO_2. Cells from exponentially growing cultures were used in all experiments.

Cloning, retroviral transduction, and selection of stable cell lines

The DNA sequence encoding Bcl-2-FLAG was amplified with PCR using the pEFFLAGBCL2pGKpuro vector, which was a kind gift of Dr. Strasser [18], as template. The PCR fragment was inserted in the retroviral vector pLNCX2 (Clontech, Palo Alto, CA) using HindIII and BglII restriction sites, and the products were verified by sequencing. To make stable retrovirus-producing cells, the packaging cell line PT67 was transfected with 5-10 □g cDNA, using Superfect reagent (Gibco BRL) according to the manufacturer's protocol. Transfected cells were grown in genecitin-containing medium and resistant colonies were selected and expanded. The stable transfected cells were then grown in medium without genecitin for 72 h, and subsequently the supernatant containing the virus was harvested and filtered through a 0.45 μm filter. After addition of hexadimethrine bromide (polybrene; Sigma, St Louis, MI) to a final concentration of 8 μg/ml, it was used to infect A431 cells. After 24 h, the virus-containing medium was removed and cells were selected in medium containing genecitin and resistant colonies were expanded. Expression of FLAG-Bcl-2 was confirmed by Western blotting using the M2 antibody that recognises the FLAG-epitope (Stratagene, La Jolla, CA).

Growth inhibition assay

For growth inhibition assays, 1 x 10^4 cells were plated into flat bottom 96-well plates (Costar, Corning, NY). After 24 h, various concentrations of the indicated drug were added and the cells were incubated for an additional 72 h. Subsequently, 10 % (v/v) of a solution of 5 mg/ml MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) was added to each well and incubated for 3 h at 37° C. Plates were centrifuged for 5 min at 1000 rpm and the medium was carefully discarded. The formed formazan crystals were dissolved in 100 μl dimethylsulfoxide (DMSO) and absorbance was determined at 540 nm using a Spectra Fluorimeter (Tecan, Salzburg, Austria). Absorbance values were expressed as the percentage of the untreated controls and the concentration of ZD1839 resulting in 50 % growth inhibition (IC50) was calculated. For C225 treatment, the percentage of maximal inhibition of growth was determined at a concentration of 10 μg/ml, since at higher concentrations of the antibody no additional growth inhibition was observed, presumably due to saturation of EGF receptors.

Clonogenic assay

Three hundred cells/well were seeded in triplicate into six well plates. Twenty-four hours later, the medium was replaced by medium containing ZD1839 or C225 at the indicated concentrations. After 72 h, the drugs were removed and cells were washed twice with PBS and allowed to grow in normal medium for 7 days. Finally, cells were stained with 0.1% crystal violet in PBS for at least 30 min at room temperature and colonies were counted. Clonogenic survival was expressed as the percentage of colony-forming units in treated cultures relative to the untreated controls.

Cell cycle analysis and cell death measurement

Cells were plated at a density of 1 x 10^5 cells per well in six wells plates (Costar, Cambridge, MA). Twenty-four hours later, the medium was replaced by medium containing the drug(s) as indicated. EGFR antagonists ZD1839 and C225 were used at concentrations of 1 μM and 5 μg/ml, respectively, and LY294002 and U0126 at a concentration of 30 μM and 10 μM, respectively. The broad-spectrum caspase inhibitor zVAD-fmk was added 1 h before treatment at a concentration of 50 μM when indicated. The cell cycle distribution was analysed of cells stained with propidium iodide (PI) [19], and the extent of cell death was determined by measuring the sub-G1 population. Apoptotic events were measured by annexin-V-FITC and 7-amino-actinomycin D (7-AAD) double staining according to the manufacturer's protocol (Nexins Research, Kattendijk, The Netherlands). All analyses were performed on a FACScalibur instrument using the CELLQuest or the ModFit 3.0 software packages (Becton Dickinson).

Caspase-3-like enzyme activity assay

Caspase-3-like enzyme activity was assayed in cellular extracts using the ApoAlert caspase-3 kit (Clontech Laboratories Inc., Palo Alto, CA) according to the manufacturer's instructions. Fluorescence was detected using a Spectra Fluorimeter equipped with a 400-nm excitation filter and a 505-nm emission filter (Tecan, Salzburg, Austria). Relative caspase activity was expressed as the level of DEVD-AFC cleavage in the treated cells compared to the level in the untreated controls.

Detection of EGFR and ErbB2 expression by flow cytometry

5x10^5 cells were harvested using trypsin and incubated for 1 h at 4 °C with 1 μg of the anti-EGFR Mab C225 (Merck) or the anti-ErbB2 Ab-2 (Neomarkers, Fremont, CA). As a control for non-specific binding, 1 μg of protein of human IgG1 lambda (Sigma) and mouse IgG1 (DAKO, Santa Barbara, CA), for the EGFR and ErbB2, respectively, were used as isotype-matched non-binding antibodies. Subsequently, cells were washed twice with ice-cold PBS containing 0.5 % BSA and incubated at 4° C in the dark for 1 h with fluorescein-isothiocyanate (FITC)-conjugated goat-anti-human or goat-anti-mouse IgG antibody, diluted 1:50 in PBS/BSA. After two washing steps with ice-cold PBS/BSA, cells were resuspended in 0.5 ml ice-cold PBS/BSA and analysed on a FACScalibur flow cytometer using CELLQuest software (Becton Dickinson, Mount View, CA). Relative expression levels were calculated as the ratio between the mean fluorescence intensity (MFI) of cells stained with the specific antibodies and the MFI of cells stained with the respective isotype-matched control antibody.

Western blotting

Cell lysates were prepared in a buffer containing 20 mM HEPES/KOH (pH 7.4), 50 mM β-glycerophosphate, 50 mM KCl, 0.2 mM EDTA, 1 % (w/v) Triton X-100 and 10 % (w/v) glycerol. A protease inhibitor cocktail (Roche, Almere, The Netherlands) and 1 mM $NaVO_3$ was freshly added to the lysis solution before each experiment. Protein concentrations were determined according to Bradford [20], using the Protein Assay Dye Reagent Solution (BioRad) with bovine

serum albumin as a standard. Cell lysates were denatured in sodium dodecyl sulfate (SDS) and equal amounts of protein were electrophoresed on 7-12 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Subsequently, membranes were blocked with 5 % non-fat dry milk for 1 h at room temperature and incubated overnight at 4° C with the appropriate primary antibodies. After incubation with horseradish peroxidase-conjugated goat-anti-mouse or goat-anti-rabbit antibodies for 1 h at room temperature, detection was performed using the enhanced chemiluminescence (ECL) reagent (Amersham). The following antibodies were used: anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-Erk, anti-Erk, anti-phospho-GSK3β anti-phospho-p90^{rsk} (all from Cell Signaling Technology, Beverly, MA), and anti-PARP (Roche, Germany).

Results

Differential effects of anti-EGFR agents on proliferation and survival in A431 and NSCLC cells

The growth of A431 cells is known to be potently inhibited by EGFR inhibitors [21-23]. In this study, we have used MTT assays to determine the effect of ZD1839 and C225 on the growth of several NSCLC cell lines in comparison to the highly sensitive A431 cells. ZD1839 induced 50 % growth inhibition in NSCLC cell lines at concentrations that were 24 to 240 times higher than the IC50 for A431 cells (Table 1). At concentrations of ZD1839 greater than 1 µM, the antiproliferative effect of ZD1839 may be mediated by actions additional to inhibition of EGFR [24]. Therefore, the growth inhibition induced at 1 µM ZD1839 compared to untreated control cells is also presented in Table 1, showing a growth inhibition of 77 % in A431 cells at this concentration, while the growth inhibition ranged from not more than 20 to 30 % in most lung cancer cells to 46 % in the A549 cell line. Similarly, C225 induced a more pronounced growth inhibition of A431 cells than of the NSCLC cells (Table 1). However, compared to ZD1839, C225 was less potent in suppressing growth of A431 cells in the MTT assay (Table 1).

In the context of a cell population, growth inhibition may result either from decreased cell proliferation or decreased cell survival. To distinguish between these possibilities, we carried out clonogenic assays on cells exposed to ZD1839 or C225 for a limited period of time. As the effect of ZD1839 is reversible upon removal [25], cells would be able to resume growth and give rise

to a colony unless their survival is compromised. ZD1839 did not reduce the clonogenic survival in any of the NSCLC cell lines, whereas treatment with ZD1839 resulted in a 50-60 % reduction of A431-derived colonies (Table 1). Similar results were obtained with C225 in this assay (Table 1).

Table 1. Comparison of protein expression levels of EGFR and ErbB2, ZD1839- and C225-induced growth inhibition and clonogenic survival.

Cell line	Relative EGFR expression[a]	Relative ErbB2 expression[a]	Growth inhibition			Clonogenic survival	
			ZD1839		C225	ZD1839	C225
			IC50[b]	% (s.d.)[c]	% (s.d.)[c]	% (s.d.)[d]	% (s.d.)[d]
A431	15	4.0	0.1	77 (± 3)	62 (± 8)	55 (± 7)	60 (± 15)
A549	2.0	3.0	2.4	46 (± 7)	49 (± 11)	97 (± 4)	97 (± 15)
H1703	4.1	6.9	7.6	29 (± 3)	35 (± 11)	n.d.[e]	n.d.
SW1573	1.8	4.5	15	20 (± 8)	19 (± 10)	95 (± 10)	108 (± 14)
H460	1.5	3.1	24	25 (± 5)	29 (± 5)	105 (± 5)	103 (± 8)

[a] Protein expression levels were analysed by flow cytometry (see Materials and Methods).
[b] Drug concentrations (μM) responsible for 50% growth inhibition in MTT assay at 72 h, calculated with data of at least three independent experiments.
[c] Growth inhibition induced by ZD1839 (1 μM) or C225 (5 μg/ml) represented as % of untreated cells, analysed by MTT assay at 72h. Mean and s.d. are shown of at least three independent experiments.
[d] Colony-forming units are expressed as a percentage of the respective untreated control. Mean and s.d. are shown of one representative experiment performed in triplicate.
[e] n.d., not determined

Effects of ZD1839 and C225 on cell cycle progression

In order to examine if the inhibitory effects observed in growth assays reflect a delay or arrest of cells in the G1/G0 phase, as shown previously [14,26], cells were treated with ZD1839 (1 μM) or C225 (5 μg/ml) for 72 h and the cell cycle progression was evaluated following propidium iodide staining and FACS analysis. An increase in the portion of cells in the G1/G0-phase of the cell cycle by 17-20% in A431 cells and 6-7% in A549 cells with a corresponding decrease in cells in S- and G2/M-phase was observed upon treatment with ZD1839 or C225 (Figure 1), correlating with the antiproliferative effects observed in these cells (see Table 1). In contrast, in H1703, SW1573, and H460 cells no change in

cell cycle distribution was detected upon treatment with the EGFR inhibitors (Figure 1), correlating with the limited antiproliferative effects observed in these cell lines at the concentrations used (see Table 1).

Interestingly, a significant ZD1839- and C225-dependent increase in the sub-G1 cell population was detected in A431 cells, but not in any of the NSCLC cell lines (Figure 1). Cells in sub-G1 population may represent apoptotic cells, suggesting that the induction of apoptosis contributes to the cytotoxic effect of the EGFR inhibitors in A431 cells.

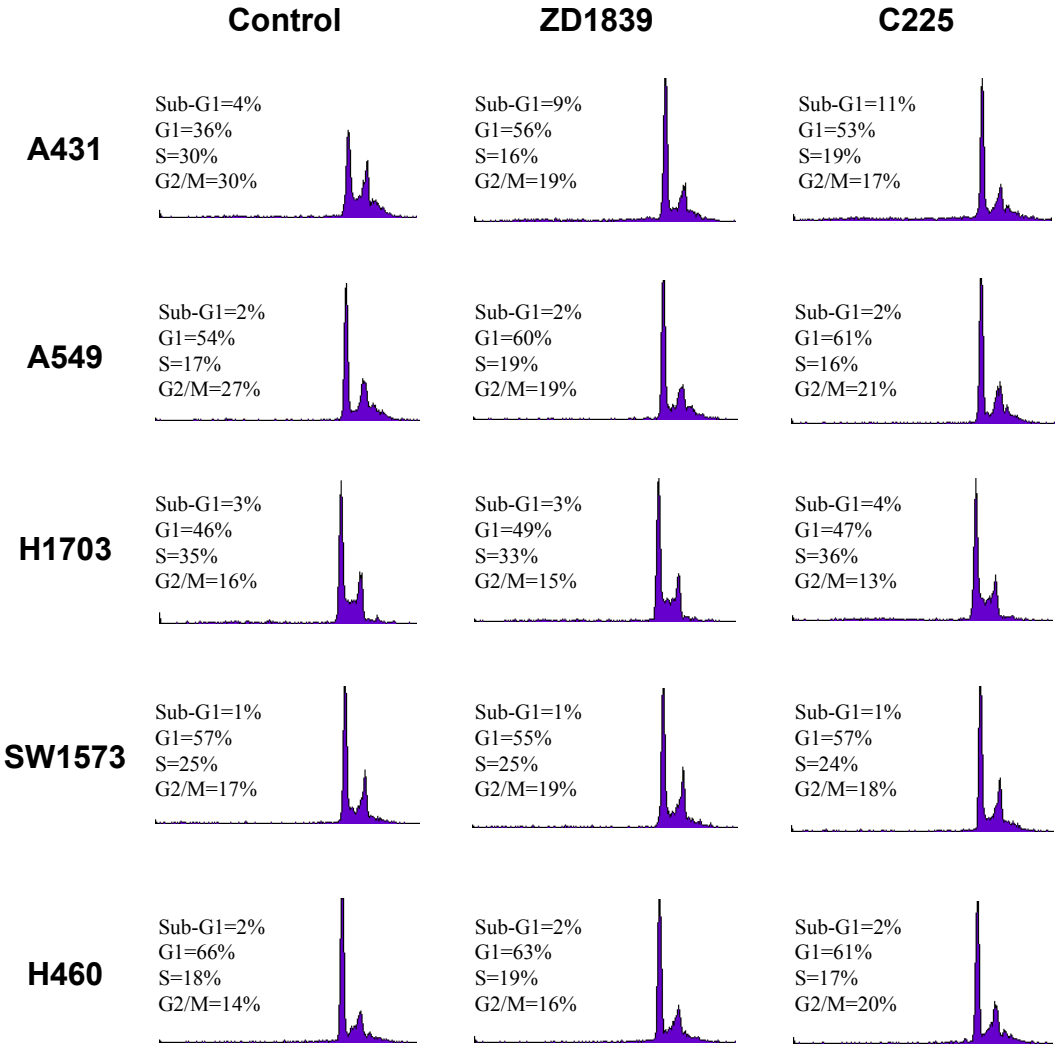


Figure 1. Cell cycle analysis of cells treated with EGFR antagonists. A431 and NSCLC cells were stained with propidium iodide (PI) after 72 h exposure to ZD1839 (1 μM) or C225 (5 μg/ml) and analysed by flow cytometry. Percentages of the total cell population in the different phases of the cell cycle were determined and included. Representative results of at least three experiments are shown.

Cytotoxity of ZD1839 in A431 cells is blocked by Bcl-2 overexpression and is partially caspase-dependent

To investigate the involvement of apoptotic cell death in ZD1839-induced cytotoxity in A431 cells, several known markers of apoptosis were evaluated. First, we examined the involvement of the anti-apoptotic protein Bcl-2, which is able to stabilise the mitochondrial membrane, thereby preventing mitochondria-dependent caspase activation [27]. It has been suggested that Bcl-2 family members have a role in apoptosis induced by EGFR-targeted agents [16,28]. To test the involvement of mitochondria in ZD1839-induced toxicity, we used retroviral transduction to generate an A431-derived cell line stably overexpressing Bcl-2. Overexpression of Bcl-2 prevented from the ZD1839-induced increase of externalised PS (phosphatidyl serine; Figure 2A), a phospholipid that is normally confined to the inner leaflet of the plasma membrane and is externalised upon induction of apoptosis. To identify late apoptotic and necrotic cells, membrane integrity was investigated by staining with 7-AAD. In addition, Bcl-2 overexpression favoured clonogenic survival of A431 cells treated with ZD1839 or C225 (Figure 2B). Interestingly, cells overexpressing Bcl-2 formed colonies that were significantly larger in size than the control vector transduced cells (Figure 2B), further illustrating the pro-survival effect of Bcl-2.

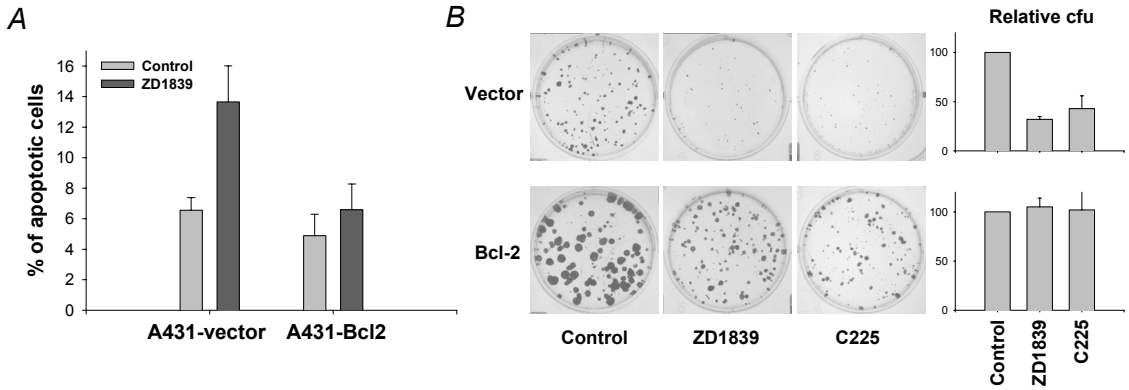


Figure 2. Overexpression of Bcl-2 protects from ZD1839-induced PS externalisation and results in clonogenic survival of A431 cells treated with ZD1839 or C225. A431 cells stably transfected with an empty retroviral vector (A431-Vector) or a vector encoding Bcl-2 (A431-Bcl-2) were treated with ZD1839 (1 μM) or C225 (5 μg/ml) for 72h. (A) The percentage of apoptotic cells represents the fraction of cells that were positively stained with annexin-V, measuring the amount of externalised PS, as analysed by flow cytometry (see also Fig. 3). (B) Clonogenic survival of A431-Bcl-2 cells, but not of A431-vector cells. Pictures were taken from representative experiments. Colony-forming units are expressed as a percentage of untreated cells and represent the mean and s.d. of at least three experiments.

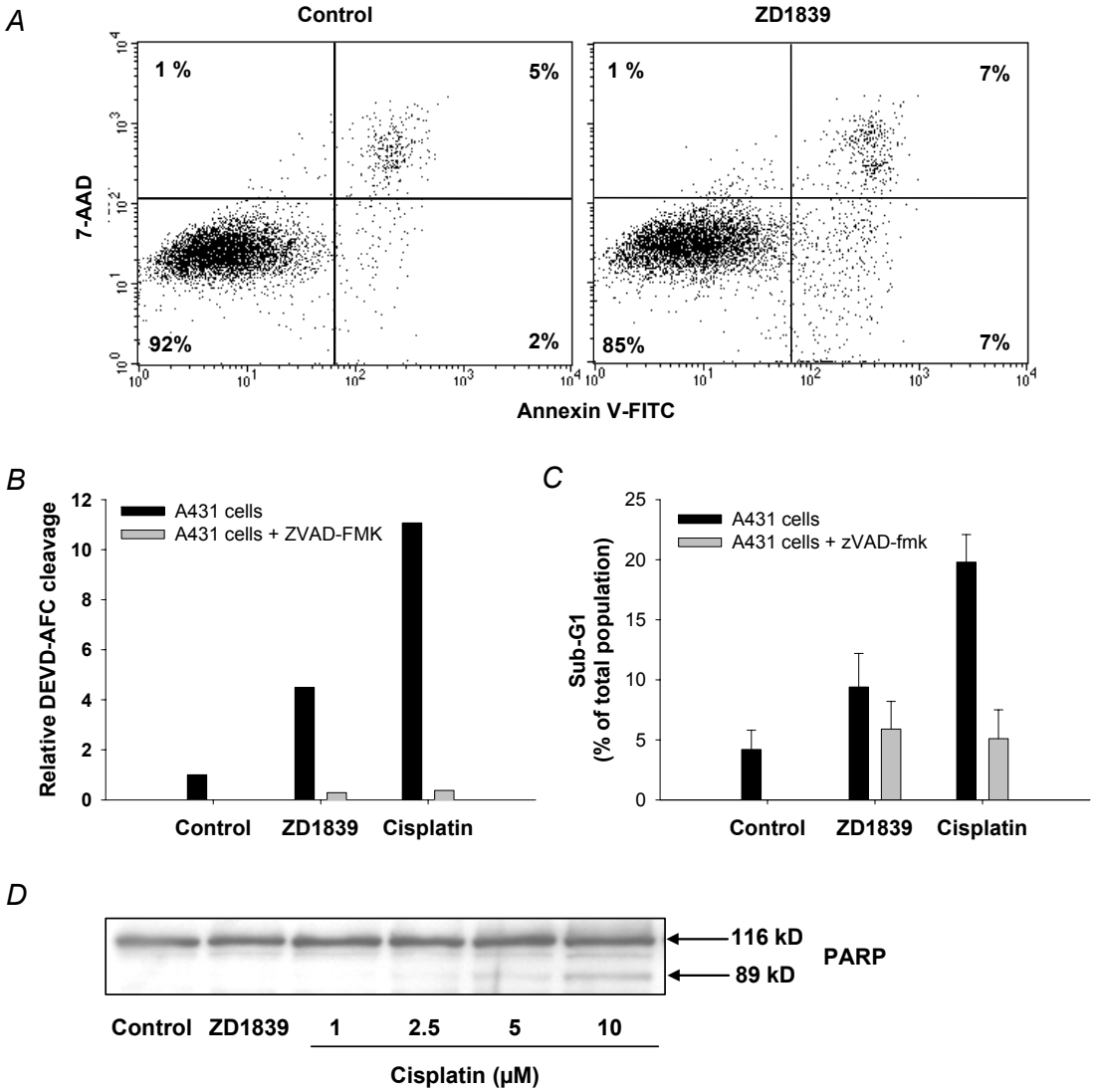


Figure 3. Partial contribution of caspase-dependent apoptosis on ZD1839-induced cytotoxity in A431 cells. (A) Cells treated with ZD1839 (1 μM) for 72h were double-stained with annexin-V/7-AAD and analysed by flow cytometry. Gate-settings distinguish between living (lower left), necrotic (upper left), early apoptotic (lower right) and late apoptotic (upper right) cells. (B) DEVD-AFC cleavage was measured in treated and untreated cells in the presence or absence of caspase inhibitors. A representative experiment is shown. (C) Cells were treated with ZD1839 (1 μM) for 72 h or cisplatin (10 μM) for 48 h in the presence or absence of zVAD-fmk. Mean and s.d. are shown from three experiments. (D) Western blot analysis of PARP cleavage in A431 cells treated with ZD1839 (1 μM) for 72 h or different concentrations of cisplatin for 48 h.

As a marker for apoptotic cell death, the exposure of PS was analysed by staining with annexin V-FITC. Upon treatment of A431 cells with ZD1839, a 2-fold increase of cells stained with annexin V-FITC was detected (Figure 3A), which is comparable to the fraction of cells in sub-G1 phase under these conditions. In ZD1839-treated NSCLC cells, no externalisation of PS was observed (data not shown), corresponding with the lack of a cytotoxic effect in these cells. To examine the role of caspases in ZD1839-induced cell death,

caspase-3-like activity was determined by measuring the cleavage of its fluorescent substrate DEVD-AFC. Caspase-3-like activity increased 4.5 times in cells treated with ZD1839 compared to 11.7 times in cisplatin-treated cells (Figure 3B), indicating that caspase-3 is activated to a lesser extent by ZD1839 than by cisplatin. As controls, ZD1839- and cisplatin-induced caspase-3-like activity was completely blocked when the broad-spectrum caspase inhibitor zVAD-fmk was added during drug exposure (Figure 3B) or when the specific caspase-3 inhibitor DEVD-CHO was added to the reaction mixture during the cleavage reaction (data not shown). Of note, zVAD-fmk decreased the proportion of cells with a hypodiploid DNA content induced by ZD1839, and completely prevented the appearance of the sub-G1 population in cisplatin-treated cells (Figure 3C). No PARP-cleavage was detected in A431 cells treated with ZD1839, whereas in control cells treated with different concentrations of cisplatin, a dose-dependent increase in PARP-cleavage was observed (Figure 3D). The lack of PARP-cleavage in ZD1839-treated cells could be due to the low amount of apoptosis that was induced, making it impossible to detect the cleaved fragment. In support of this, no PARP-cleavage was detected in cells treated with 1 μM cisplatin (Fig. 3D), a concentration that induces a sub-G1 population (11%) that was similar to that resulting from ZD1839-treatment (data not shown). Taken together, these results demonstrate that the induction of caspase-dependent apoptosis contributes, at least in part, to the cytotoxity induced by ZD1839 in A431 cells.

Lack of correlation between EGFR or ErbB2 expression levels and ZD1839 and C225 toxicity in NSCLC cells

It is not yet known whether sensitivity to EGFR inhibitors depends on the amount of EGFR [23,29,30] and/or ErbB2 [22,23,26] expressed by cells. The NSCLC cell lines used in this study expressed moderate levels of EGFR when compared to the control A431 cell line that expresses very high levels of EGFR (Table 1). Expression of ErbB2, on the other hand, was similar in all cell lines (Table 1). As previously reported [21-23], the highly EGFR expressing A431 cells were extremely sensitive to the EGFR inhibitors, but neither EGFR nor ErbB2 expression correlated with the growth inhibitory effect of ZD1839 or C225 within the panel of NSCLC cell lines.

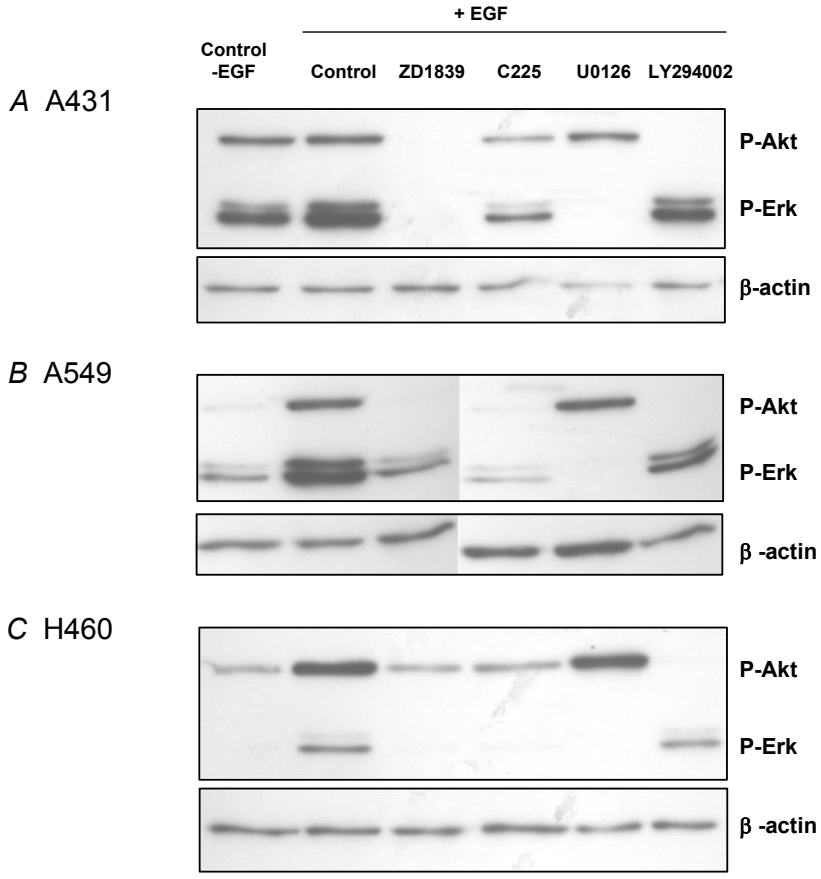


Figure 4. Persistent activity of Erk or Akt in NSCLC cell lines, but not in A431 cells. (A-C) A431, A549, and H460 cells were serum-starved overnight and treated with ZD1839 (1 μM), C225 (5 μg/ml), U0126 (10 μM), or LY294002 (30 μM) for 2 h prior to exposure to 10 ng/ml EGF for 5 minutes. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were immunostained for phosphorylated Erk and Akt. As control for protein loading, membranes were stripped and reprobed with antibodies recognising β-actin.

Effects of ZD1839 and C225 on kinase signalling downstream of EGFR

EGFR signalling is transduced through two main kinase pathways, involving MEK/Erk and PI3K/Akt [31]. Intrinsic activity of these pathways can potentially circumvent EGFR inhibition. Phosphorylation of Erk and Akt was analysed to determine the activation status of the two pathways upon anti-EGFR treatment. The sensitive A431 cell line and two NSCLC cell lines (A549 and H460) were treated with the EGFR antagonists ZD1839 or C225, the MEK inhibitor U0126, or the PI3K inhibitor LY294002 prior to stimulation with EGF. Cell extracts were subsequently subjected to Western blot analysis. Non-stimulated A431 control cells displayed strong bands corresponding to phosphorylated Erk and Akt, and stimulation with EGF resulted even in a slight increase in phosphorylation (Figure 4A, lane 1 and 2). Phosphorylation of Erk

and Akt was abolished in ZD1839-treated A431 cells (Figure 4A, lane 3), which is in agreement with previous observations by others in these cells [21,23]. Compared to ZD1839, C225 only partially reduced phosphorylation of these downstream molecules in A431 cells (Figure 4A, lane 4). This may explain the smaller effect of the anti-EGFR antibody on growth, as shown earlier in the MTT assay and suggests less effective EGFR kinase inhibition by C225 than by ZD1839 in A431 cells. As expected, the MEK inhibitor (U0126) and the PI3K inhibitor (LY294002) specifically inhibited Erk or Akt phosphorylation, respectively (Figure 4A, lane 5 and 6). Unlike A431 cells, phosphorylated Erk, but not Akt, was present in serum-deprived A549 cells (Figure 4B, lane 1), while the reverse was found in H460 cells, having phosphorylated Akt and non-phosphorylated Erk (Figure 4C, lane 1), suggesting intrinsic activity of one of the kinase pathways in these cells. Incubation with EGF resulted in a significant increase of phosphorylated Erk and Akt in both cell lines (Figure 4B and C, lane 2), demonstrating the functionality of the EGFR pathway in these cells. Treatment with ZD1839 or C225 resulted in the decrease of phosphorylation of Erk and Akt to the levels seen in the untreated controls, with activated Erk (in A549 cells) or Akt (in H460 cells) still detectable (Figure 4B and C, lanes 3 and 4). However, in both lung cancer cell lines, treatment with U0126 or LY294002 completely abrogated Erk or Akt phosphorylation, respectively (Figure 4B and C, lanes 5 and 6).

These results clearly indicate that the Erk or Akt kinase pathways are constitutively active and are not effectively blocked by EGFR antagonists in the NSCLC cell lines A549 and H460, respectively.

Dose-dependent inhibition of EGF-induced signalling via Erk and Akt kinase pathways by ZD1839

To further substantiate the latter findings, we extended our analysis to other NSCLC cell lines and treated with different concentrations of ZD1839. In addition to Erk and Akt phosphorylation, the cell extracts were analysed for phosphorylation of $p90^{rsk}$ and GSK3β, which are downstream substrates of Erk and Akt, respectively [32,33]. A dose-dependent decrease of Erk and Akt phosphorylation was observed in A431 cells, coinciding with decreased phosphorylation of $p90^{rsk}$ and GSK3β (Figure 5A), indicating that both kinase pathways were blocked by ZD1839 in these cells. In A549 (Figure 5B) and

SW1573 cells (Figure 5C), phosphorylation of Erk and p90^{rsk} were only partially inhibited, but showed a dose-dependent decline of Akt and GSK3β phosphorylation. In contrast, H460 cells showed dose-dependent reduction of Erk and p90^{rsk} phosphorylation, but only in part of Akt and GSK3β phosphorylation (Figure 5D). In the H1703 cell line, no effects of ZD1839 were observed on the phosphorylation status of Erk, p90^{rsk}, Akt and GSK3β (Figure 5E). The dose-dependent effect shows that inhibition of the Erk and Akt kinase pathways by ZD1839 is the consequence of EGFR blocking. Furthermore, these data confirm that at least one of the kinase pathways involving Erk and Akt is persistently active in the presence of ZD1839 in the NSCLC cells, whereas both pathways are effectively blocked in A431 cells.

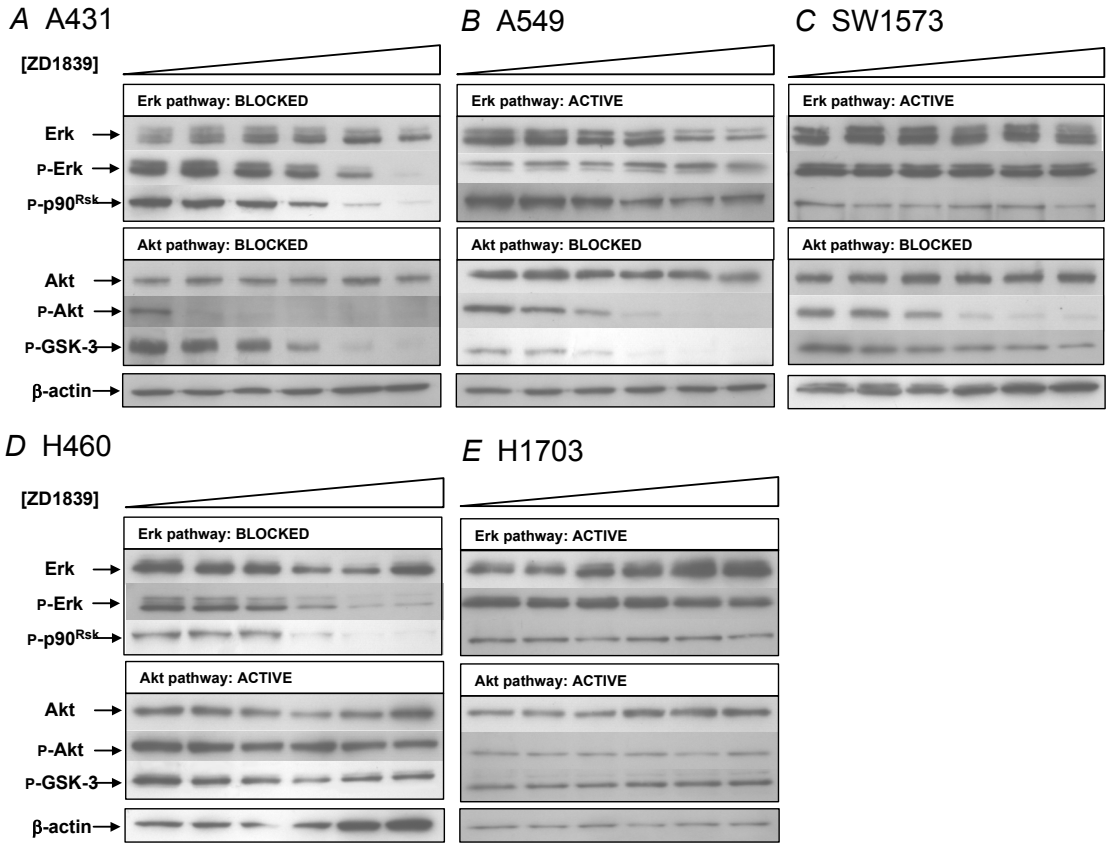


Figure 5. Dose-dependent inhibition of Erk and Akt kinase pathways by ZD1839. Serum-starved A431 cells (A) and NSCLC cells (B-E) were treated with different concentrations of ZD1839 (0, 0.05, 0.1, 0.25, 0.5, and 1 μM) for 2 h prior to stimulation with 10 ng/ml EGF for 5 min. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were immunostained for phosphorylated and total Erk and Akt, phosphorylated p90^{rsk} and phosphorylated GSK3β. As a control for equal protein loading, membranes were stripped and reprobed with antibodies recognising β-actin. BLOCKED: effective inhibition of the Erk or Akt pathway by ZD1839; ACTIVE: persistent activity of the Erk or Akt pathway after treatment with ZD1839.

Simultaneous inhibition of the MEK and PI3K pathways results in effective inhibition of growth and apoptosis

The above results suggest that persistent activity of the MEK and/or PI3K pathway contributes to the resistance of NSCLC cells to EGFR antagonists. To independently investigate the role of MEK and PI3K in proliferation, we determined the effect of U0126 and LY294002 on the growth of A431, A549, and H460 cells. As single agents, both inhibitors induced a dose-dependent growth inhibition that was similar in all cell lines in a 72 h MTT assay (Fig. 6A-C), demonstrating a role of both kinase pathways in the proliferation of these cells. When cells were treated with a combination of U0126 and LY294002, the effect on growth was at least additive in all cell lines (Fig. 6A-C), suggesting an independent role of the MEK and PI3K pathway in proliferation. When compared with U0126 and LY294002 as single agents or in combination, ZD1839 was more effective to inhibit the growth of A431 cells (Fig. 6A), indicating that ZD1839 is more potent to inhibit its target than U0126 or LY294002, and that A431 cells are largely dependent on EGFR activity for their growth. In contrast, treatment of NSCLC cells with U0126 and/or LY294002 produced a more pronounced inhibition of cell growth than treatment with ZD1839 (Fig. 6B-C). This can be explained by the fact that ZD1839 specifically blocks EGFR-dependent signalling, while activation of downstream pathways in the presence of EGFR inhibitors is still possible by enhanced activity of proteins such as Ras or PI3K [34,35], or via other receptors that may be activated by growth factors or hormones in the serum [22]. U0126 and LY294002 directly inhibit the MEK or PI3K pathway, thereby blocking EGFR-dependent and -independent signalling and inducing stronger antiproliferative effects. In addition, despite the selectivity of the MEK and PI3K inhibitors, we cannot rule out their potential effects on other targets involved in proliferation, in particular at higher concentrations [36].

In addition, we used U0126 and LY294002 to determine the role of the MEK and PI3K pathways on apoptosis in A431, A549, and H460 cells. As shown earlier, ZD1839 induced a 2- to 3-fold increase of A431 cells with hypodiploid DNA, while no effect was observed in the A549 or H460 cell line (Fig. 1; Fig. 6D-F). Neither U0126 nor LY294002 induced changes in the sub-G1 population as single agent in any of the cell lines, while the combination of both agents

induced an increase in the sub-G1 population in A431 as well as the lung cancer cells to a level that was similar to the sub-G1 population induced by ZD1839 in A431 cells (Fig. 6D-F). These data indicate that the inhibition of the MEK and the PI3K pathway in A431 cells by ZD1839 can explain the cell death induced in these cells, and support the view that the persistent activity of at least one of these pathways may protect the NSCLC cells from ZD1839-induced apoptosis.

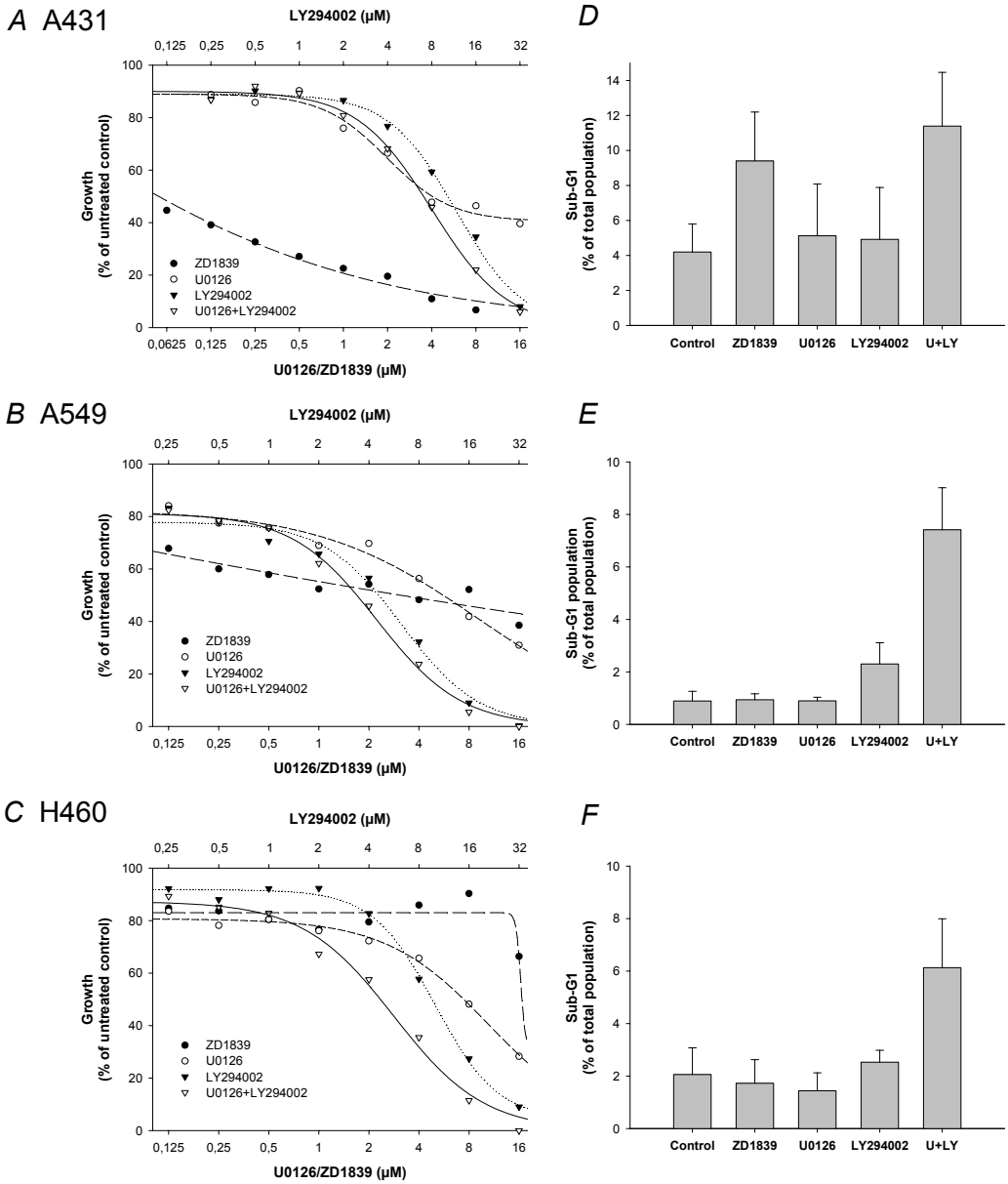


Figure 6. Effective growth inhibition and induction of apoptosis in cells with inhibited MEK and PI3K. (A-C) Growth curves of A431, A549, and H460 cells treated with different concentrations of ZD1839, U0126, LY294002, or a combination of U0126 and LY294002, with a ratio of 1:2. Growth was represented as % of untreated cells, analysed by MTT assay at 72h. The plots were fitted using Sigma plot software (four-parameter logistic curve). Similar results were obtained in at least three independent experiments. (D-E) Analysis of the sub-G1 population induced by ZD1839 (1 μM), U0126 (10 μM), LY294002 (30 μM), or a combination of U0126 and LY294002. A431, A549, and H460 cells were treated with the kinase inhibitors for 72 h, subsequently stained with propidium iodide, and analysed by flow cytometry.

Discussion

In this study, we show that treatment of NSCLC cell lines with EGFR inhibitors induce differential antiproliferative effects, although the effect on growth is limited when compared to the control A431 cell line. In the highly sensitive A431 cell line and to a lesser extent also in A549 cells, the growth inhibitory effect of the EGFR antagonists correlates with an arrest of cells in the G1/G0-phase of the cell cycle, while the cell cycle is unaffected in the more resistant lung cancer cell lines (Fig. 1). These results are consistent with earlier reports, showing that antiproliferative effects of ZD1839 or C225 correlate with a G1/G0-phase cell cycle arrest in cell lines derived from breast and head and neck tumors [14,26].

We demonstrate that treatment with EGFR-inhibitors induces a modest apoptotic response in A431 cells, but does not affect the survival of NSCLC cells (Table 1; Fig. 3). Comparable amounts of apoptosis have been reported for several other cell lines treated with ZD1839 or C225 [12,14], while effective induction of apoptosis by EGFR-targeted agents has been reported in only a few cell lines [16,17]. In line with an earlier report demonstrating that ZD1839 induces apoptosis by activating the pro-apoptotic Bcl-2-family member BAD in mammary cells [16], we found that overexpression of Bcl-2 prevents ZD1839- and C225-induced cell death in A431 cells. We further show that caspases contribute, at least in part, to the cytotoxity of ZD1839 in A431 cells.

We investigated molecular differences that may underlie the variable sensitivity of A431 and NSCLC cells to anti-EGFR agents. It has been reported that cells with high expression of EGFR [21,23] or ErbB2 [22,23,26] are particularly sensitive to ZD1839, suggesting that abundant expression of EGFR or ErbB2 is required to modulate sensitivity. However, Moasser *et al* also demonstrated that high endogenous EGFR expression *per se* does not determine sensitivity to EGFR inhibitors [23]. Since EGFR and ErbB2 are part of a large signalling network [4], a number of factors rather than expression levels of the receptors alone may determine the sensitivity of a cell to EGFR inhibitors. In support of this view, we found that the sensitivity to the anti-EGFR agents within the panel of lung cancer cells differed significantly, although all expressed similar, moderate protein levels of EGFR compared to the highly EGFR-expressing A431 cell line,

whereas the ErbB2 expression levels were similar in all cell lines, including A431. This indicates that neither EGFR nor ErbB2 expression levels correlate with sensitivity to EGFR inhibitors in NSCLC cells.

Next, we determined the activation status of the MEK and the PI3K pathway, two major intracellular signalling pathways activated by the EGFR. Sensitivity to EGFR-inhibitors was found to correlate with persistent activity of these pathways in presence of ZD1839 in the panel of NSCLC cell lines, whereas they are effectively blocked in the A431 cell line (Fig. 5). In line with this, Brognard *et al* recently showed that Erk and Akt were constitutively active in the majority of NSCLC cell lines deprived of serum [37,38]. Moreover, our results showing more effective antiproliferative effects when both pathways are blocked, indicate that the MEK and PI3K pathways may independently contribute to the proliferation of these cells (Fig. 6B-C). These data are in line with previous evidence showing that intrinsically active Erk [30] and Akt [23] correlate with reduced antiproliferative activity of ZD1839 in several epithelial tumor cell lines, while the growth inhibitory effect of the HER2-targeted Mab herceptin is prevented in breast cancer cells ectopically overexpressing an active mutant of Akt [39].

In addition to their contribution to cell proliferation, persistently active Erk and Akt pathways may protect cells from apoptosis induced by EGFR-targeted agents. Earlier reports demonstrated that cells transfected with active mutants of members of the MEK/Erk or PI3K/Akt pathway bypassed apoptosis induced by ZD1839 [16] or herceptin [39]. We found that chemical inhibition of either MEK or PI3K did not generate apoptosis of A431 or NSCLC cells (Fig. 6C-F), which was recently also shown in the A549 and other NSCLC cell lines [37,38]. In contrast, disabling the MEK and the PI3K pathway resulted in the induction of a modest apoptotic response in the A431 and lung cancer cell lines, similar to the amount of apoptosis induced by ZD1839 in A431 cells (Fig. 6C-F). These data further support the idea that persistently active MEK and PI3K pathways, which are present in the NSCLC cell lines, account for the unaffected survival of these cells in the presence of anti-EGFR agents.

The limited effect observed *in vitro* contrasts with some *in vivo* studies reporting complete regressions of A431-derived tumors in mice treated with ZD1839 or C225, and 70-80% growth inhibition of A549-derived tumors in

ZD1839-treated mice [15,24,40]. Mechanisms that are only active *in vivo* may explain the more effective antitumor activity *in vivo* compared to the limited antiproliferative effects *in vitro*, such as observed in A549 cells. First, C225 and ZD1839 can inhibit angiogenesis, which has been proposed to be the result of reduced secretion of angiogenesis factors [41-45], while anti-EGFR agents can also directly inhibit the growth and cell-cell interaction of endothelial cells [24,46]. Second, C225 was shown to inhibit metastasis of bladder carcinoma xenografts [44], demonstrating the implication of EGFR signalling in cell migration and invasion [47,48].

The results presented here, showing that constitutively active Erk and Akt could contribute to resistance to anti-EGFR treatment, may have important clinical relevance. To select for patients that may benefit from anti-EGFR therapy, it may be important to identify tumors that do not carry intrinsically active Erk or Akt. Immunohistochemical analysis of tumors for activated Erk and Akt may predict response to ZD1839 and C225, and will give more insight if constitutively activated Erk and/or Akt correlate with higher resistance to anti-EGFR treatment in patients. In fact, activated Erk has been correlated with EGFR activity in tumors [22,49] and is downregulated in the skin from cancer patients treated with ZD1839 [50], suggesting that activated Erk can be used as a marker for EGFR activity *in vivo*. On the other hand, anti-EGFR treatment in combination with specific inhibitors targeting kinase pathways via MEK or PI3K, some of which are now tested in preclinical and clinical studies [51], might result in additional antitumor effect in some types of NSCLC.

References

1. Salomon, DS *et al.* Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* **19**, 183-232 (1995).

2. Rusch, V *et al.* Overexpression of the epidermal growth factor receptor and its ligand transforming growth factor alpha is frequent in resectable non-small cell lung cancer but does not predict tumor progression. *Clin Cancer Res* **3**, 515-522 (1997).

3. Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **103**, 211-225 (2000).

4. Yarden, Y *et al.* Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2**, 127-137 (2001).

5. Ciardiello, F *et al.* A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. *Clin Cancer Res* **7**, 2958-2970 (2001).

6. Woodburn, JR. The epidermal growth factor receptor and its inhibition in cancer therapy. *Pharmacol Ther* **82**, 241-250 (1999).

7. Waksal, HW. Role of an anti-epidermal growth factor receptor in treating cancer. *Cancer Metastasis Rev* **18**, 427-436 (1999).

8. Barker, AJ *et al.* Studies leading to the identification of ZD1839 (IRESSA): an orally active, selective epidermal growth factor receptor tyrosine kinase inhibitor targeted to the treatment of cancer. *Bioorg Med Chem Lett* **11**, 1911-1914 (2001).

9. Ciardiello, F. Epidermal growth factor receptor tyrosine kinase inhibitors as anticancer agents. *Drugs* **60**, 25-32 (2000).

10. Arteaga, CL *et al.* Tyrosine kinase inhibitors-ZD1839 (Iressa). *Curr Opin Oncol* **13**, 491-498 (2001).

11. Ranson, M *et al.* ZD1839, a Selective Oral Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitor, Is Well Tolerated and Active in Patients With Solid, Malignant Tumors: Results of a Phase I Trial. *J Clin Oncol* **20**, 2240-2250 (2002).

12. Ciardiello, F *et al.* Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. *Clin Cancer Res* **5**, 909-916 (1999).

13. Ciardiello, F *et al.* Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* **6**, 2053-2063 (2000).

14. Huang, SM *et al.* Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. *Cancer Res* **59**, 1935-1940 (1999).

15. Sirotnak, FM *et al.* Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* **6**, 4885-4892 (2000).

16. Gilmore, AP *et al.* Activation of BAD by Therapeutic Inhibition of Epidermal Growth Factor Receptor and Transactivation by Insulin-like Growth Factor Receptor. *J Biol Chem* **277**, 27643-27650 (2002).

17. Wu, X *et al.* Apoptosis induced by an anti-epidermal growth factor receptor monoclonal antibody in a human colorectal carcinoma cell line and its delay by insulin. *J Clin Invest* **95**, 1897-1905 (1995).

18. Ekert, PG *et al.* Inhibition of apoptosis and clonogenic survival of cells expressing crmA variants: optimal caspase substrates are not necessarily optimal inhibitors. *EMBO J* **18**, 330-338 (1999).

19. Nicoletti, I *et al.* A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* **139**, 271-279 (1991).

20. Bradford, MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-254 (1976).

21. Albanell, J *et al.* Activated extracellular signal-regulated kinases: association with epidermal growth factor receptor/transforming growth factor alpha expression in head

and neck squamous carcinoma and inhibition by anti-epidermal growth factor receptor treatments. *Cancer Res* **61**, 6500-6510 (2001).

22. Anderson, NG *et al.* ZD1839 (Iressa), a novel epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, potently inhibits the growth of EGFR-positive cancer cell lines with or without erbB2 overexpression. *Int J Cancer* **94**, 774-782 (2001).

23. Moasser, MM *et al.* The tyrosine kinase inhibitor ZD1839 ("Iressa") inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. *Cancer Res* **61**, 7184-7188 (2001).

24. Wakeling, AE *et al.* ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. *Cancer Res* **62**, 5749-5754 (2002).

25. Woodburn, JR *et al.* ZD1839, an epidermal growth factor tyrosine kinase inhibitor selected for clinical development. **Proc. Am. Ac. Cancer Res.**, Abstract-#633 (1997).

26. Moulder, SL *et al.* Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells in vitro and in vivo. *Cancer Res* **61**, 8887-8895 (2001).

27. Adams, JM *et al.* The Bcl-2 protein family: arbiters of cell survival. *Science* **281**, 1322-1326 (1998).

28. Mandal, M *et al.* Nuclear targeting of Bax during apoptosis in human colorectal cancer cells. *Oncogene* **17**, 999-1007 (1998).

29. Bos, M *et al.* PD153035, a tyrosine kinase inhibitor, prevents epidermal growth factor receptor activation and inhibits growth of cancer cells in a receptor number-dependent manner. *Clin Cancer Res* **3**, 2099-2106 (1997).

30. Magne, N *et al.* Influence of epidermal growth factor receptor (EGFR), p53 and intrinsic MAP kinase pathway status of tumour cells on the antiproliferative effect of ZD1839 ('Iressa'). *Br J Cancer* **86**, 1518-1523 (2002).

31. Olayioye, MA *et al.* The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J* **19**, 3159-3167 (2000).

32. Cross, DA *et al.* Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785-789 (1995).

33. Dalby, KN *et al.* Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90rsk that are inducible by MAPK. *J Biol Chem* **273**, 1496-1505 (1998).

34. Hoshino, R *et al.* Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* **18**, 813-822 (1999).

35. Simpson, L *et al.* PTEN: life as a tumor suppressor. *Exp Cell Res* **264**, 29-41 (2001).

36. Davies, SP *et al.* Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* **351**, 95-105 (2000).

37. Brognard, J *et al.* Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. *Cancer Res* **61**, 3986-3997 (2001).

38. Brognard, J *et al.* Variable apoptotic response of NSCLC cells to inhibition of the MEK/ERK pathway by small molecules or dominant negative mutants. *Cell Death Differ* **9**, 893-904 (2002).

39. Yakes, FM *et al.* Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt Is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. *Cancer Res* **62**, 4132-4141 (2002).

40. Goldstein, NI *et al.* Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. *Clin Cancer Res* **1**, 1311-1318 (1995).

41. Bruns, CJ *et al.* Epidermal growth factor receptor blockade with C225 plus gemcitabine results in regression of human pancreatic carcinoma growing orthotopically in nude mice by antiangiogenic mechanisms. *Clin Cancer Res* **6**, 1936-1948 (2000).

42. Ciardiello, F *et al.* Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res* **7**, 1459-1465 (2001).

43. Hirata, A *et al.* ZD1839 (Iressa) Induces Antiangiogenic Effects through Inhibition of Epidermal Growth Factor Receptor Tyrosine Kinase. *Cancer Res* **62**, 2554-2560 (2002).

44. Perrotte, P *et al.* Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res* **5**, 257-265 (1999).

45. Petit, AM *et al.* Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* **151**, 1523-1530 (1997).

46. Huang, SM *et al.* Modulation of radiation response and tumor-induced angiogenesis after epidermal growth factor receptor inhibition by ZD1839 (Iressa). *Cancer Res* **62**, 4300-4306 (2002).

47. Damstrup, L *et al.* In vitro invasion of small-cell lung cancer cell lines correlates with expression of epidermal growth factor receptor. *Br J Cancer* **78**, 631-640 (1998).

48. Verbeek, BS *et al.* Overexpression of EGFR and c-erbB2 causes enhanced cell migration in human breast cancer cells and NIH3T3 fibroblasts. *FEBS Lett* **425**, 145-150 (1998).

49. Albanell, J *et al.* Pharmacodynamic studies with the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839. *Semin Oncol* **28**, 56-66 (2001).

50. Albanell, J *et al.* Pharmacodynamic studies of the epidermal growth factor receptor inhibitor ZD1839 in skin from cancer patients: histopathologic and molecular consequences of receptor inhibition. *J Clin Oncol* **20**, 110-124 (2002).

51. Dy, GK *et al.* Novel targets for lung cancer therapy: part I. *J Clin Oncol* **20**, 2881-2894 (2002).

Chapter 3

Enhanced cytotoxicity induced by gefitinib and specific inhibitors of the Ras or Phosphatidyl Inositol-3 Kinase pathways in non-small cell lung cancer cells.

Maarten L Janmaat
José A Rodriguez
Mariëlle Gallegos-Ruiz
Frank AE Kruyt
Giuseppe Giaccone

International Journal of Cancer 2006, 118: 209-14

Abstract

In this study, we have characterized a panel of NSCLC cell lines with differential sensitivity to gefitinib for activating mutations in *egfr*, *pik3ca*, and *k-ras*, and basal protein expression levels of PTEN. The *egfr* mutant NSCLC cell line H1650 as well as the *egfr* wild type cell lines H292 and A431 were highly sensitive to gefitinib treatment, indicating that other factors determine gefitinib sensitivity in *egfr* wild type cells. Activating *k-ras* mutations were specifically detected in gefitinib-resistant cells, suggesting that the occurrence of *k-ras* mutations is correlated with resistance to EGFR antagonists. No *pik3ca* mutations were detected within the panel of cell lines, and PTEN protein expression levels did not correlate with gefitinib sensitivity. Gefitinib effectively blocked Akt and Erk phosphorylation in two gefitinib-sensitive NSCLC cell lines, further supporting our previous findings that persistent activity of the PI3K/Akt and/or Ras/Erk pathways is associated with gefitinib-resistance of NSCLC cell lines. Gefitinib-resistant NSCLC cell lines, showing EGFR-independent activity of the PI3K/Akt or Ras/Erk pathways, were treated with gefitinib in combination with specific inhibitors of mTOR, P13K, Ras, and MEK. Additive cytotoxicity was observed in A549 cells co-treated with gefitinib and the MEK inhibitor U0126 or the farnesyl transferase inhibitor SCH66336 and in H460 cells treated with gefitinib and the PI3K inhibitor LY294002, but not in H460 cells treated with gefitinib and rapamycin. These data suggest that combination treatment of NSCLC cells with gefitinib and specific inhibitors of the PI3K/Akt and Ras/Erk pathways may provide a successful strategy.

Introduction

The EGFR[II] (ErbB1, HER1) is the prototypic member of the ErbB family of RTKs, which further consists of ErbB2-4 (HER2-4). The ErbB receptors share a similar protein structure, consisting of an extracellular ligand binding domain, a single transmembrane domain and an intracellular C-terminal domain with tyrosine kinase activity. Upon specific binding of EGF-like ligands to the extracellular domain, ErbB receptors dimerize, either as homodimers or as heterodimers, and undergo autophosphorylation at specific tyrosine residues within the intracellular domain. These phosphorylated tyrosines serve as docking sites for adapter molecules such as Grb2 and the p85 subunit of PI3K, which activate downstream signalling pathways. These pathways, including the Ras/MAPK and Akt/mTOR kinase cascades, in turn, regulate transcription factors and other proteins involved in cell proliferation, survival, motility, and differentiation [1] (Figure 1). Aberrantly high EGFR activity is common in several tumor types, including NSCLC, and correlates with a more aggressive disease, resistance to chemotherapy, and poor patient prognosis.

Monoclonal antibodies to the extracellular domain of EGFR, such as cetuximab, and EGFR tyrosine kinase inhibitors, including gefitinib and erlotinib, specifically block EGFR activity. In preclinical studies, these agents have been demonstrated to inhibit cell proliferation and tumor growth and, in some models, to induce cell death and tumor shrinkage [2-5]. Many preclinical studies have demonstrated anti-tumor effects of cetuximab and gefitinib in combination with other anticancer therapies, in particular chemotherapeutic agents and radiation [2,3,5]. In clinical settings, single agent anti-EGFR therapy resulted in significant anti-tumor activity in several cancer types, including in 10-20 % of NSCLC patients [6]. As a result of this, gefitinib and erlotinib has been registered for the second and third line treatment of NSCLC in the US and other countries. In contrast to the promising preclinical data, no additional effects of EGFR inhibitors have been observed in four large phase III clinical trials in NSCLC patients, in which gefitinib or erlotinib were added to standard chemotherapy [6-8].

Recently, a strong correlation has been reported between somatic mutations in the *egfr* gene and dramatic clinical response to gefitinib in patients

with NSCLC [9,10]. Conversely, we and others have shown that persistent activity of the Ras/Erk and PI3K/Akt kinase pathways contributes to resistance of NSCLC cells to EGFR inhibitors [4,11,12]. Growth factor independent activity of EGFR-downstream pathways may be due to several tumor-associated alterations, including activating mutations in the *k-ras* [13] or *pik3ca* [14] genes, or loss of the tumor suppressor *pten* [15].

In this study, we have characterized a panel of NSCLC cell lines for specific mutations in the EGFR, k-ras, and PI3K genes and we evaluated basal protein expression and activation status of EGFR pathway components, including PTEN. The relationship between the presence of these alterations and the ability of gefitinib to inhibit the EGFR-downstream pathways was assessed. Finally, in those cell lines showing EGFR-independent activity of the PI3K/Akt or Ras/Erk pathways, we investigated the effect of gefitinib in combination with specific inhibitors of mTOR, PI3K, Ras, and MEK.

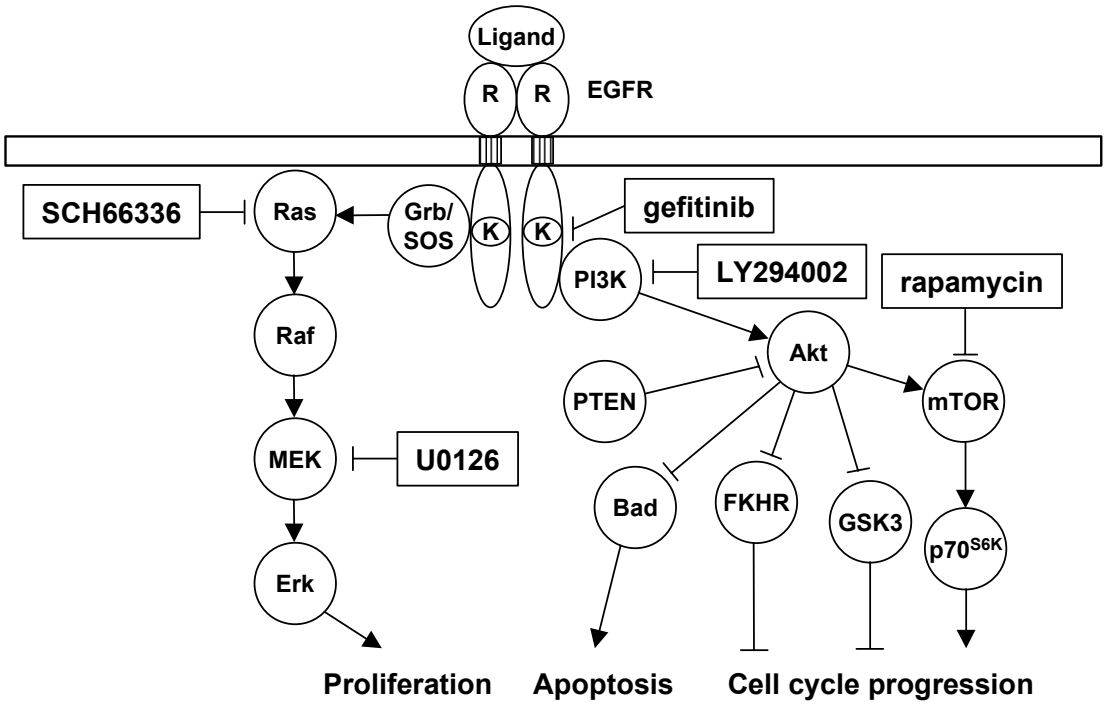


Figure 1. The EGF receptor pathway and the action sites of specific kinase- and farnesyl-transferase-inhibitors. Schematic representation of major components of the EGF receptor pathway. Drugs that have been used in this study interfere at specific sites within this pathway, as indicated. See the text for further details.

Materials and Methods

Cells and culture conditions

RPMI 1640 (containing 2 mM L-glutamine) and DMEM were used as culture media and were supplemented with 10 % heat-inactivated fetal calf serum (Gibco BRL, Life technology, Breda, The Netherlands), 50 iu/ml penicillin and 50 □g/ml streptomycin. The human NSCLC cell lines NCI-H460 (H460), NCI-H1703 (H1703), NCI-H292 (H292), NCI-H1650 (H1650) and A549 cells were cultured in RPMI 1640, the human NSCLC cell line SW1573 and the epidermoid carcinoma cell line A431 were cultured in DMEM. Cells were grown at 37° C in a humidified atmosphere with 5 % CO_2. Cells from exponentially growing cultures were used in all experiments.

Table 1. Primers used for nested amplification of genomic DNA sequences of EGFR, PIK3CA, and k-ras.

Gene, exon		Forward	Reverse
EGFR, exon 18	Int[a]	CTGAGGTGACCCTTGTCTCT	CCATGAGAGGCCCTA
	Ext[b]	ATGGTGAGGGCTGAGGTGAC	TCCCCACCAGACCATGAGAG
EGFR, exon 19	Int	TCTCACAATTGCCAGTTAAC	TGAGGTTCAGAGCCATGGAC
	Ext	GTGGCACCATCTCACAATTG	AAGGTGGGCCTGAGGTTCAG
EGFR, exon 20	Int	CCCCTCCTTCTGGCCACCAT	ATCCTGGCTCCTTATCTCCC
	Ext	GTCCATGTGCCCCTCCTTC	GCATGTGAGGATCCTGGCTC
EGFR, exon 21	Int	AATTCGGATGCAGAGCTTCT	CTGGTGTCAGGAAAATGCTG
	Ext	ATGACCCTGAATTCGGATG	AGCCTGGTCCCTGGTGTCAG
EGFR, exon 22	Int	GTCCAGAGTGAGTTAACTTT	CTTGGCCTCAGTACAAACTC
	Ext	TCGTAATTAGGTCCAGAGTG	ATAAAAGCCAGCTTGGCCTC
EGFR, exon 23	Int	AAAGCAAGGGATTGTGATTG	AGCCACCAAGGCTCAGCTAG
	Ext	AATGATGACTAAAGCAAGGG	GGCTAAGAGCAGCCACCAAG
EGFR, exon 24	Int	CATCTTTATCATTTCTTCCA	ATGAGATGCTCATTAGAGGG
	Ext	TAAGCAATGCCATCTTTATC	ATGAGATGCTCATTAGAGGG
PIK3CA, exon 9	Int	GAGGGGAAAAATATGACAAA	ATTTTTTCTGTAATAAAGAA
	Ext	TGTGAATCCAGAGGGGAAAA	AAATTCAGTTATTTTTTCTG
PIK3CA, exon 20.1	Int	GCTCCAAACTGACCAAACTG	CTGTTTAATTGTGTGGAAGA
	Ext	AACATCATTTGCTCCAAACT	TCAATGCATGCTGTTTAATT
PIK3CA, exon 20.2	Int	GACAACAAAAATGGATTGGA	TCACTTTTTCCTTCTCCATC
	Ext	ATGGTGGCTGGACAACAAAA	AAAAAAACCATCACTTTTTC
k-Ras, exon 1	Int	AGTCACATTTTCATTATTTT	AGAAACCTTTATCTGTATCAAAGAATG
	Ext	GTTCTAATATAGTCACATTT	ACTCATGAAAATGGTCAGAGAAACCTTTAT
k-Ras, exon 2	Int	GTGCACTGTAATAATCCAGA	ACTCCTTAATGTCAGCTTAT
	Ext	GAAGTAAAAGGTGCACTGTA	AACTATAATTACTCCTTAAT

DNA isolation, PCR and primers

Total genomic DNA was isolated from cell lines using DNA STAT-60™ (Tel-Test Inc, Friendswood, TX), according to the manufacturer's protocol. Nested PCRs were carried out using the external and internal primers detailed in Table 1. Sequencing of PCR products was performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), the M13 primers 5'-TGTAAAACGACGGCCAGT-3' (forward) and 5'-

CAGGAAACAGCTATGACC-3' (reverse) and the ABI PRISM™ 310 Genetic analyzer (Applied Biosystems). The mutations found were confirmed by sequencing independent PCR products.

Chemicals

The EGFR-TKI gefitinib (Iressa™, ZD1839) was kindly provided by AstraZeneca (Macclesfield, UK) as a pure substance, which was diluted in dimethylsulfoxide (DMSO) to a stock concentration of 20 mM. The farnesyl transferase inhibitor SCH66336 (provided by Schering-Plough, Kenilworth, NJ), and the kinase inhibitors rapamycin (LC Laboratories, Woburn, MA), U0126 and LY294002 (both from Cell Signalling Technology, Beverly, MA) were also diluted in DMSO.

MTT assays

For cellular survival assays 5 x 10^3 cells were plated into flat bottom 96-well plates (Costar, Corning, NY). After 24 h, the growth medium was replaced by medium containing 0.5 % fetal calf serum when indicated. Subsequently, various concentrations of the indicated drugs were added and the cells were incubated for an additional 72 h. Next, 10 % (v/v) of a solution of 5 mg/ml MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) was added to each well and incubated for 3 h at 37° C. Plates were centrifuged for 5 min at 1200 rpm and the medium was carefully discarded. The formed formazan crystals were dissolved in 100 µl DMSO, and absorbance was determined at 540 nm using a Spectra Fluorimeter (Tecan, Salzburg, Austria). Absorbance values were expressed as the percentage of the untreated controls. The concentration of gefitinib resulting in 50 % growth inhibition (IC50) was calculated as described before [4].

Western blotting

SDS-PAGE and Western Blotting was performed as previously described [4]. In short, whole cell lysates were denatured in sample buffer containing SDS, and equal amounts of total protein were separated on 8-15 % SDS-poly-acrylamide gels and transferred to nitrocellulose membranes. After blocking with 5 % non-fat dry milk, the membranes were incubated overnight at 4° C with the primary antibodies as indicated. The following antibodies were used: anti-PTEN, anti-phospho-EGFR (Tyr1068), anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-Erk, anti-Erk, anti-phospho-p70s6K (all from Cell Signaling Technology), anti-EGFR (Ab-12) (Neomarkers, Fremont, CA), and anti-□-actin (Sigma, Zwijndrecht, the Netherlands). The following day, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies, and detection was performed using enhanced chemiluminescence reagent (Amersham Biosciences, Amersham, UK).

Statistics

ANOVA analysis was used to evaluate potential differences between the single agent treatment groups and the combination treatment groups. *P*-values resulted from the use of the Tamhane's T2 test (assumed unequal variances).

RESULTS

Characterization of egfr, k-ras, and pik3ca gene status and PTEN protein levels in a panel of cell lines

Growth factor-independent activation of the Akt and Erk pathways can be due to activating mutations in the *k-ras* [13] or the *pik3ca* [14] genes, respectively. Recently, it has been demonstrated that specific activating mutations in the *egfr* gene are predictive for a clinical response to gefitinib in NSCLC patients [9,10]. To investigate the implications of EGFR, k-ras, and PIK3CA mutations on gefitinib-response, we characterized a panel of NSCLC cell lines and the gefitinib-sensitive A431 vulval carcinoma cell line for activating mutations in these genes. Only the H1650 cell line, included as a positive control [16], was found to have a mutation in the *egfr* gene, whereas all other tested cell lines, including the gefitinib-sensitive A431 and H292 cells, were *egfr* wild type (Table 2). *K-ras* mutations were found in three of the tested cell lines, which were all found to be relatively resistant to treatment with gefitinib (Table 2). The presence of mutations in the *k-ras* gene was associated with a lack of Erk response to gefitinib treatment in A549 and SW1573 cells, which harbor a mutation in codon 12 of *k-ras* (Table 2). This may have been expected due to growth factor-independent activity of mutated k-Ras protein. In contrast, gefitinib efficiently reduced Erk phosphorylation in the H460 cell line, which harbors a mutation in codon 61 of *k-ras*, indicating that *k-ras* mutations do not always result in EGFR-independent Erk activity. None of the cell lines harbored mutations in hotspot regions of the *pik3ca* gene (Table 2), encoding the p110α catalytic subunit of PI3K [14]. Depletion of the tumor suppressor *pten* can also increase growth factor-independent activity of the PI3K/Akt pathway [15] and causes resistance of tumor cell lines, including NSCLC cells, to gefitinib [11,17]. As analyzed by Western blotting, basal protein expression levels of PTEN varied between the different cell lines (Figure 2). However, no relationship was found between PTEN expression levels and gefitinib sensitivity or the ability of gefitinib to inhibit the PI3K/Akt pathway (Table 2).

Table 2. Characterization of EGFR, k-ras, and PIK3CA genes in a panel of cell lines in comparison to gefitinib sensitivity and p-Erk and p-Akt response to gefitinib.

Cell line	IC50 gefitinib (μM)[1]	gefitinib response[1]		gene status			PTEN expression[5]
		p-Erk[2]	p-Akt	EGFR exons 18-24	PIK3CA exons 9+20	K-ras exon 1+2	
A431	<0.1	Blocked[3]	Blocked	Wt[4]	Wt	Wt	0.26
H1650	<0.1	Blocked	Blocked	Mut (DelE746-A750)	Wt	Wt	0.03
H292	<0.1	Blocked	Blocked	Wt	Wt	Wt	0.60
A549	2.4	Active	Blocked	Wt	Wt	Mut (G12S)	0.44
H1703	7.6	Active	Active	Wt	Wt	Wt	0.15
SW1573	15	Active	Blocked	Wt	Wt	Mut (G12C)	0.25
H460	24	Blocked	Active	Wt	Wt	Mut (G61H)	1.00

[1] IC50 concentrations for gefitinib and p-Erk and p-Akt response to gefitinib have been partially reported previously [4].

[2] *p-Erk*, phosphorylated Erk; *p-Akt*, phosphorylated Akt

[3] *Blocked,* effective inhibition of phosphorylated Erk or Akt by gefitinib; *Active,* persistent phosphorylation of Erk or Akt after treatment with gefitinib.

[4] *Wt*, wild-type; *Mut (changed amino acid)*, mutated; *Del*, deletion.

[5] PTEN protein expression levels relative to H460, analyzed by quantifying the Western Blot data shown in Figure 2, normalized to β-actin.

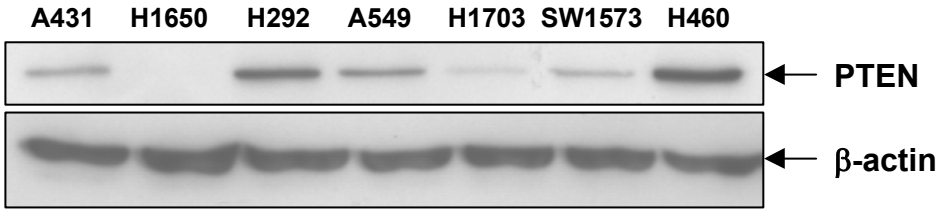


Figure 2. Characterization of basal PTEN protein expression levels in a panel of NSCLC cell lines with different sensitivity to gefitinib. Total protein expression levels of PTEN were analysed by Western blotting using a polyclonal antibody, raised against human PTEN-derived peptides. Blots were reprobed for β-actin as a control for equal protein loading.

The Akt and Erk pathways are effectively blocked by EGFR antagonists in gefitinib-sensitive NSCLC cell lines

Previously, we reported that persistent activity of the PI3K/Akt and/or Ras/MEK/Erk pathways is associated with higher resistance of NSCLC cell lines to EGFR antagonists [4]. We provide here further support for our previous finding by extending the panel of cell lines to include two gefitinib-sensitive NSCLC cell lines, H292 and H1650 (Table 2). Treatment of these cells with gefitinib effectively inhibited EGF-induced phosphorylation of EGFR, Akt and Erk (Figure 3*A*, *B*; lane 3) in contrast to gefitinib-resistant cells (Table 2). In gefitinib-sensitive

cell lines, the EGFR-specific antagonistic antibody C225 inhibited EGF-induced EGFR phosphorylation, but did not affect Akt and Erk phosphorylation, or blocked it less effectively than gefitinib (data not shown). As expected, the PI3K inhibitor LY294002 and the MEK inhibitor U0126 completely abrogated Akt or Erk phosphorylation, respectively (Figure 3*A*, *B*; lanes 4 and 5).

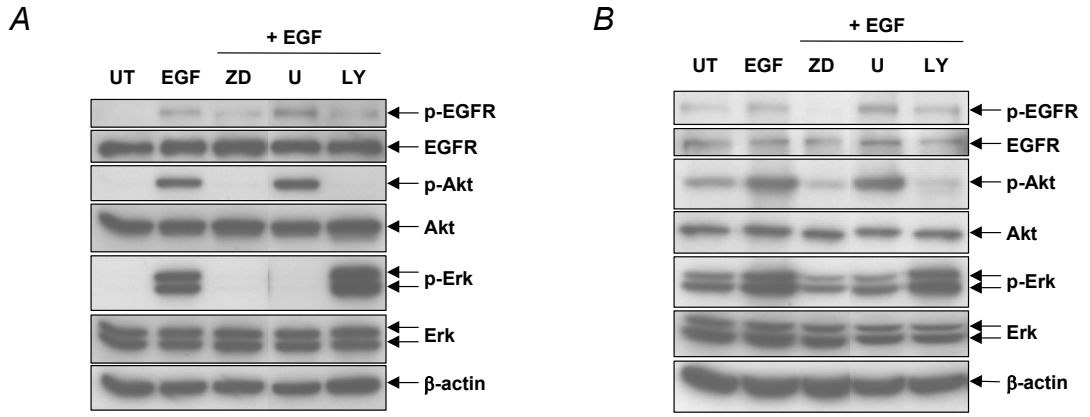


Figure 3. Gefitinib blocks the Akt- and Erk-pathways in gefitinib-sensitive NSCLC cell lines. Western blot analysis of gefitinib-sensitive NSCLC cell lines H292 (*A*) and H1650 (*B*) that were treated with ZD1839 (1 μM), U0126 (10 μM), or LY294002 (30 μM) for 2 h before exposure to EGF (10 ng/ml) for 5 min. Protein levels of total and phosphorylated (p) EGFR, Akt, and Erk were analysed.

Enhanced cytotoxic effect of gefitinib with the PI3K inhibitor LY294002 but not with rapamycin in H460 cells

We hypothesized that if EGFR-independent activity of the PI3K/Akt pathway contributes to gefitinib-resistance in NSCLC cell lines [4] (table 2), anti-EGFR treatment in combination with specific inhibitors of the PI3K pathway may result in additional cytotoxic effects in NSCLC cell lines such as H460, in which the PI3K pathway is not effectively blocked by gefitinib. To test this hypothesis, growth inhibition was determined in H460 cells treated with gefitinib in combination with the PI3K inhibitor LY294002 or the mTOR inhibitor rapamycin, which act at different sites in the PI3K/Akt pathway (Figure 1). Combined treatment of two concentrations of LY294002 and gefitinib resulted in reduced growth of H460 cells compared to treatment with single agents (Figure 4*A*). As serum may activate growth factor pathways parallel to the EGFR pathway, bypassing the inhibition of EGFR, Ras, or MEK, growth inhibition assays were also carried out under low (0.5 %) serum conditions. Co-treatment with gefitinib and the two different concentrations LY294002 reduced the growth of H460 cells under

these conditions (Figure 4*A*). Although this reduction of cell growth was not significantly different from treatment with single agents, these data suggest that gefitinib and LY294002 have at least an additive interaction. In contrast to LY294002, addition of rapamycin to gefitinib did not result in enhanced cytotoxicity in H460 cells grown under high or low serum conditions (Figure 4*B*).

To verify that the observed growth effects of gefitinib in combination with rapamycin or LY294002 were due to the inhibition of PI3K and/or mTOR in addition to EGFR, we analyzed the activation status of downstream components by Western blotting. As shown before [4], gefitinib completely inhibited basal and EGF-induced phosphorylation of EGFR and Erk, whereas EGF-induced phosphorylation of Akt and p70s6K, downstream of Akt, was only inhibited to levels observed in untreated cells (Figure 4*C*, lanes 3 and 4). Treatment with rapamycin completely and specifically eradicated phosphorylation of the mTOR substrate p70s6K, but not of Erk or the upstream kinases EGFR and Akt (Figure 4*C*, lanes 5 and 6). Addition of gefitinib to rapamycin removed EGF-induced phosphorylation of EGFR and Erk in addition to p70s6K, but not of Akt (Figure 4*C*, lanes 7 and 8). The PI3K inhibitor LY294002 specifically blocked phosphorylation of Akt and p70s6K (Figure 4*C*, lanes 9 and 10), while addition of gefitinib to LY294002 effectively inhibited EGFR, Akt, Erk, and p70s6K phosphorylation (Figure 4*C*, lanes 11 and 12). Interestingly, EGF stimulation induced a rapid downregulation of total EGFR in H460 cells, which was efficiently blocked by gefitinib (Figure 4*C*).

Additive cytotoxic effects of gefitinib with inhibitors of the Ras/MEK pathway in A549 cells

In addition to persistent activity of the PI3K pathway, EGFR-independent activity of the Ras/Erk pathway contributes to gefitinib-resistance in NSCLC cell lines [4]. Therefore, we hypothesized that anti-EGFR treatment in combination with specific inhibitors of the Ras/Erk kinase pathway may result in additional cytotoxic effects in NSCLC cell lines, such as A549 and SW1573 cells, that maintain Erk phosphorylation after gefitinib treatment (see Table 2). To test this hypothesis, cell growth was determined in A549 and SW1573 cells treated with gefitinib in combination with the specific MEK inhibitor U0126 or the farnesyl transferase inhibitor SCH66336, which inhibits farnesylation and activation of

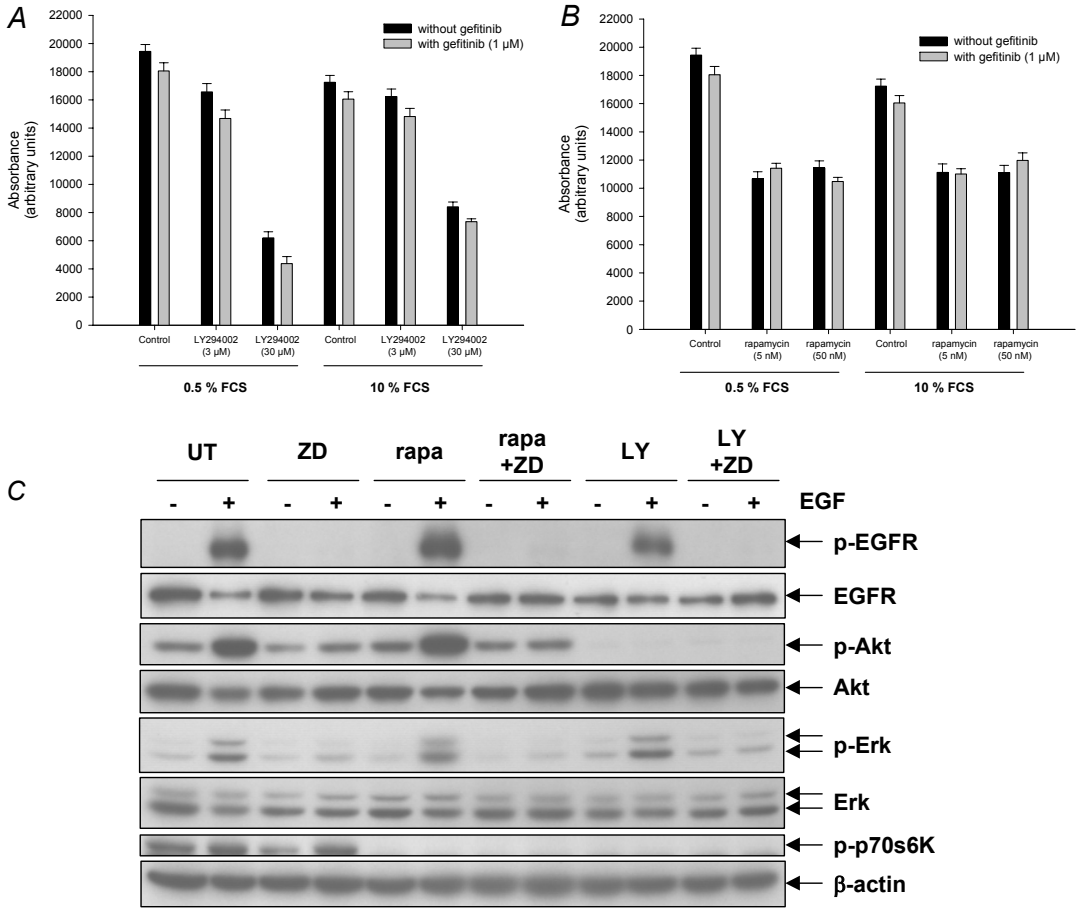


Figure 4. Combined inhibition of the EGF receptor and the PI3K/Akt/mTOR pathway in H460 cells. The NSCLC-derived H460 cell line was treated with two different concentrations of *A,* LY294002 or *B,* rapamycin with or without gefitinib (1 µM) for 72 h. Experiments were carried out under low (0.5 %) and high (10 %) fetal calf serum conditions. The bars represent the mean absorbance (arbitrary units) and standard error obtained in least three independent MTT assays carried out in triplo (*, $P \leq 0.05$; **, $P \leq 0.01$, Tamhane's T2 test). *C,* Western blot analysis of H460 cells treated with gefitinib (ZD; 1 µM), rapamycin (rapa; 50 nM), LY294002 (LY; 30 µM), or combinations for 2 h before exposure to EGF (10 ng/ml) for 5 min. Protein levels of total and phosphorylated (p) EGFR, Akt, and Erk were analysed.

Ras proteins [18]. Addition of gefitinib to two different concentrations of U0126 (Figure 5*A*) or SCH66336 (Figure 5*B*) reduced the growth of A549 cells compared to treatment with single agents under high as well as low serum conditions. Statistically significant effects were observed only in cells treated with 8 µM U0126 and gefitinib (1µM) under low serum conditions and in cells treated with 1 µM SCH66336 and gefitinib (1 µM) under high serum conditions, indicating that the efficacy is dependent on the used conditions. In contrast to A549 cells, no enhanced effects of gefitinib on U0126- or SCH66336- induced cytotoxicity were observed in SW1573 cells under both serum conditions (data not shown).

To confirm that the observed cytotoxic effects were due to inhibition of Erk phosphorylation, the effects of SCH66336 and U0126 on the activation status of EGFR downstream pathway components were examined. U0126 specifically and effectively inhibited Erk phosphorylation and did not affect the phosphorylation of EGFR or Akt (Figure 5*C*, lanes 9 and 10). Unexpectedly, SCH66336 induced Erk phosphorylation to a similar extent as in EGF-treated cells, while stimulation of SCH66336-treated cells with EGF even further increased Erk phosphorylation (Figure 5*C*, lanes 5 and 6). Co-treatment of cells with gefitinib did not affect SCH66336-induced Erk phosphorylation, but blocked the EGF-enhanced Erk phosphorylation (Figure 5*C*, lanes 7 and 8). SCH66336-induced Erk phosphorylation was also observed in SW1573 cells (data not shown).

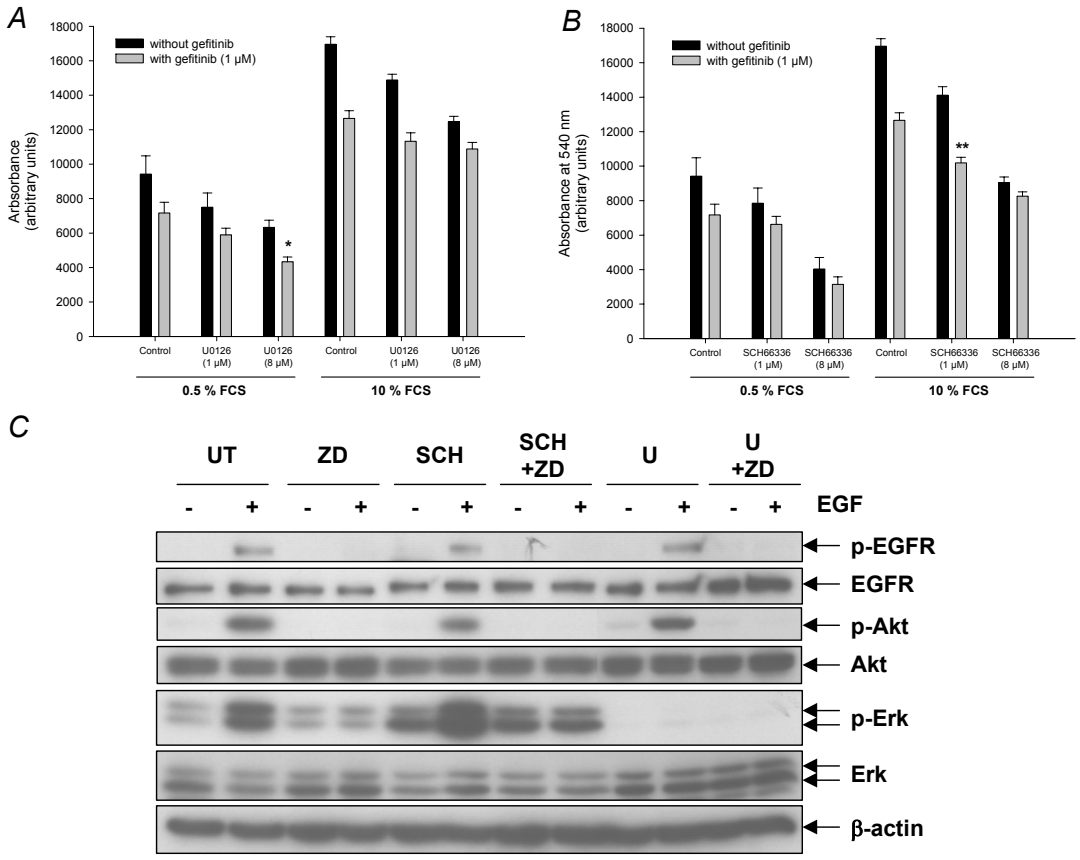


Figure 5. Combined inhibition of the EGF receptor and the Ras/MEK pathway in A549 cells. *A, B,* A549 cells were treated with two different concentrations of *A,* U0126 or *B,* SCH66336 with or without gefitinib (1 µM) for 72 h. Experiments were carried out under low (0.5 %) and high (10 %) fetal calf serum conditions. The bars represent the mean absorbance (arbitrary units) and standard error obtained in least three independent MTT assays carried out in triplo (*, $P \leq 0.05$; **, $P \leq 0.01$, Tamhane's T2 test). *C,* Western blot analysis of A549 cells treated with gefitinib (ZD; 1 µM), SCH66336 (SCH; 8 µM), U0126 (U; 8 µM), or combinations for 2 h before exposure to EGF (10 ng/ml) for 5 min. Protein levels of total and phosphorylated (p) EGFR, Akt, and Erk were analysed.

Discussion

We have previously reported that persistent activity of the PI3K/Akt and/or Ras/Erk pathways is associated with gefitinib-resistance of NSCLC cell lines and that simultaneous inhibition of PI3K/Akt and MEK/Erk reduces tumor cell survival more effectively than inhibition of each pathway alone [4]. Here, we provide further support for these findings by demonstrating that gefitinib effectively blocks Akt and Erk phosphorylation in two gefitinib-sensitive NSCLC cell lines.

We carried out a detailed mutational and expression analysis of key genes and proteins that may be involved in modulating the response to gefitinib. As expected, we observed that the NSCLC cell line H1650, harboring a mutation in *egfr*, is sensitive to gefitinib treatment. However, we found that the other gefitinib-sensitive NSCLC cell line, H292, and A431 cells do not harbor activating mutations in the *egfr* gene. This finding suggests that other factors determine the strong EGFR-dependency in these EGFR wild type cell lines. In fact, the gefitinib-sensitive A431 cell line is known to harbor about 30 copies of the *egfr* gene within its genome [19], resulting in excessive EGFR protein expression [4]. H292 cells also express relatively high levels of EGFR (data not shown), although this cell line has not been investigated for *egfr* gene amplifications. To select patients that benefit from anti-EGFR therapy in the clinic, it will be important to identify markers that can predict the sensitivity of *egfr* wild type tumors. For example, it could be possible that within the subset of *egfr* wild type tumors EGFR expression levels are predictive for outcome.

Mutational analysis of genes encoding EGFR downstream components revealed that activating *k-ras* mutations were only detected in gefitinib-resistant NSCLC cell lines, whereas no mutations were observed in gefitinib-sensitive cell lines. This, in contrast to other reports [20], suggests that occurrence of *k-ras* mutations is correlated with resistance to EGFR antagonists. None of the cell lines harbored mutations in hotspot regions of the *pik3ca* gene, encoding the p110α catalytic subunit of PI3K, which have been reported to occur at low frequency in NSCLC [14]. In contrast to other reports [11,17], our data suggest that absence of the tumor suppressor *pten* is not necessarily a factor of resistance to gefitinib, at least in the presence of an *egfr* mutation, as we observed a lack of

PTEN protein expression in the gefitinib-sensitive, *egfr*-mutant H1650 cell line. Treatment with gefitinib efficiently inhibited Akt phosphorylation in these cells, showing that Akt phosphorylation is still EGFR-dependent in these PTEN-deficient cells. Conversely, Akt phosphorylation was inefficiently inhibited by gefitinib in H460 cells, which express relatively high PTEN protein levels.

Further supporting our view that simultaneous inhibition of Akt and Erk results in stronger cytotoxic effects than inhibition of each pathway alone, we demonstrate here that treatment with gefitinib in combination with specific Ras, MEK or PI3K inhibitors results in enhanced cytotoxic effects in gefitinib-resistant NSCLC cell lines. For example, co-treatment of H460 cells treated with gefitinib and the PI3K inhibitor LY294002 or co-treatment of A549 cells with gefitinib and the MEK inhibitor U0126 resulted in enhanced cytotoxicity. In contrast to the PI3K inhibitor LY294002, the mTOR inhibitor rapamycin, acting downstream of PI3K and Akt, did not enhance the cytotoxic effects of gefitinib in H460 cells, although rapamycin effectively inhibited the phosphorylation of the mTOR substrate p70s6K. These data indicate that enhancement of gefitinib-induced cytotoxicity in H460 cells requires additional inhibition of Akt but is independent of inhibition of the downstream mTOR pathway. On the other hand, treatment of A549 cells with the farnesyl transferase inhibitor SCH66336, thought to inhibit Ras activity [18], enhanced gefitinib-induced cytotoxicity. However, the additive interaction between SCH66336 and gefitinib appears to be independent from Erk inhibition, as SCH66336 unexpectedly induced an increase of Erk phosphorylation in this cell line, in contrast to previously observed SCH66336-induced decrease of Erk phosphorylation in glioma cell lines and Ras-transformed Rat2 fibroblasts [21,22]. The exact molecular mechanism underlying the additive interaction between gefitinib and SCH66336 requires further investigation.

Although the enhanced cytotoxicity of gefitinib in combination with Ras, MEK or PI3K inhibitors appears to be largely independent of factors present in the serum, it may be affected by cell type-specific differences. Thus, similar effects were observed in H460 and A549 cells in high or low serum conditions, but no additive effects of combination treatments were noted in SW1573 cells.

Together, our data suggest that treatment of NSCLC cell lines with combinations of gefitinib and specific inhibitors of the PI3K/Akt or Ras/Erk

pathways is a successful strategy. Moreover, to our knowledge we are the first to show that an EGFR antagonist enhances the cytotoxicy of a farnesyl transferase inhibitor in NSCLC cells. Although the efficacy and tolerability of the combinations have to be tested more extensively in preclinical models, our results can be meaningful in the clinic, as many specific signal transduction inhibitors are being developed for clinical use. In fact, small molecule inhibitors that specifically target various steps of the PI3K-Akt and Ras-MAPK pathways have entered clinical trials, including farnesyl transferase inhibitors, MEK inhibitors and rapamycin derivatives [23,24].

References

1. Yarden, Y *et al.* Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2**, 127-37 (2001).

2. Sirotnak, FM *et al.* Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* **6**, 4885-92 (2000).

3. Ciardiello, F *et al.* Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* **6**, 2053-63 (2000).

4. Janmaat, ML *et al.* Response to Epidermal Growth Factor Receptor Inhibitors in Non-Small Cell Lung Cancer Cells: Limited Antiproliferative Effects and Absence of Apoptosis Associated with Persistent Activity of Extracellular Signal-regulated Kinase or Akt Kinase Pathways. *Clin Cancer Res* **9**, 2316-2326 (2003).

5. Baselga, J *et al.* Antitumor effects of doxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies. *J Natl Cancer Inst* **85**, 1327-33 (1993).

6. Herbst, RS *et al.* Gefitinib--a novel targeted approach to treating cancer. *Nat Rev Cancer* **4**, 956-65 (2004).

7. Herbst, RS *et al.* Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 2. *J Clin Oncol* **22**, 785-94 (2004).

8. Giaccone, G *et al.* Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial--INTACT 1. *J Clin Oncol* **22**, 777-84 (2004).

9. Paez, JG *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304**, 1497-500 (2004).

10. Lynch, TJ *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* **350**, 2129-39 (2004).

11. She, QB *et al.* Resistance to gefitinib in PTEN-null HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacologic modulation of

constitutive phosphatidylinositol 3'-kinase/Akt pathway signaling. *Clin Cancer Res* **9**, 4340-6 (2003).

12. Magne, N *et al.* Influence of epidermal growth factor receptor (EGFR), p53 and intrinsic MAP kinase pathway status of tumour cells on the antiproliferative effect of ZD1839 ("Iressa"). *Br J Cancer* **86**, 1518-23 (2002).

13. Rodenhuis, S *et al.* Mutational activation of the K-ras oncogene. A possible pathogenetic factor in adenocarcinoma of the lung. *N Engl J Med* **317**, 929-35 (1987).

14. Samuels, Y *et al.* High frequency of mutations of the PIK3CA gene in human cancers. *Science* **304**, 554 (2004).

15. Stambolic, V *et al.* Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95**, 29-39 (1998).

16. Sordella, R *et al.* Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science* **305**, 1163-7 (2004).

17. Bianco, R *et al.* Loss of PTEN/MMAC1/TEP in EGF receptor-expressing tumor cells counteracts the antitumor action of EGFR tyrosine kinase inhibitors. *Oncogene* **22**, 2812-22 (2003).

18. Liu, M *et al.* Antitumor activity of SCH 66336, an orally bioavailable tricyclic inhibitor of farnesyl protein transferase, in human tumor xenograft models and wap-ras transgenic mice. *Cancer Res* **58**, 4947-56 (1998).

19. Merlino, GT *et al.* Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. *Science* **224**, 417-9 (1984).

20. Suzuki, T *et al.* The sensitivity of lung cancer cell lines to the EGFR-selective tyrosine kinase inhibitor ZD1839 ('Iressa') is not related to the expression of EGFR or HER-2 or to K-ras gene status. *Lung Cancer* **42**, 35-41 (2003).

21. Glass, TL *et al.* Inhibition of cell growth in human glioblastoma cell lines by farnesyltransferase inhibitor SCH66336. *Neuro-oncol* **2**, 151-8 (2000).

22. Brassard, DL *et al.* Inhibitors of farnesyl protein transferase and MEK1,2 induce apoptosis in fibroblasts transformed with farnesylated but not geranylgeranylated H-Ras. *Exp Cell Res* **273**, 138-46 (2002).

23. Huang, S *et al.* Targeting mTOR signaling for cancer therapy. *Curr Opin Pharmacol* **3**, 371-7 (2003).

24. Sebolt-Leopold, JS *et al.* Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat Rev Cancer* **4**, 937-47 (2004).

Chapter 4

Predictive factors for outcome in a phase II study of gefitinib in second-line treatment of advanced esophageal cancer patients.

Maarten L Janmaat
Mariëlle I Gallegos-Ruiz
José A Rodriguez
Gerrit A Meijer
Walter L Vervenne
Dick J Richel
Cees Van Groeningen
Giuseppe Giaccone

Journal of Clinical Oncology 2006, 24: 1612-9

Abstract

Purpose: The efficacy of the EGFR tyrosine kinase inhibitor gefitinib was assessed in a phase II study in patients with advanced esophageal cancer. Several biological features were investigated as potential markers of gefitinib activity.

Patients and methods: Patients with advanced esophageal cancer, who had failed one line of prior chemotherapy, were given gefitinib 500 mg/day. Response was evaluated every 8 weeks. Tumor material obtained before gefitinib treatment was investigated for gene mutations in *EGFR*, *k-ras*, and *PIK3CA*, protein expression levels of EGFR, p-Akt, and p-Erk, and *EGFR* gene amplification.

Results: Of the 36 enrolled patients, one achieved a partial response (2.8%), 10 had stable disease (27.8%), 17 (47.2%) progressed on treatment, while 8 (22.2%) were not evaluable for response. The progression-free survival was 59 days and the median overall survival was 164 days. Although *EGFR* or *PIK3CA* mutations were absent, *k-ras* mutations were found in two patients with progressive disease. High *EGFR* gene copy number was identified in two patients experiencing partial response or progressive disease. A higher disease control rate (response plus stable disease) was observed in female (P=0.038) and in patients with SCC (P=0.013) or high EGFR expression (P=0.002).

Conclusion: Gefitinib has a modest activity in second-line treatment of advanced esophageal cancer. However, the patient outcome was significantly better in female and in patients demonstrating high EGFR expression or SCC histology. The selection of esophageal cancer patients for future studies with EGFR-TKIs based on the level of EGFR expression in their tumors or SCC histology should be considered.

Introduction

Esophageal cancer is a disease with a high mortality, and is the fastest growing malignancy in the United States [1]. The survival depends on the stage of the disease. Surgical resection is the treatment of choice for early lesions. In recent years, possibly due to introduction of broader use of flexible endoscopes, tumors that are confined to mucosa and submucosa are more frequently diagnosed [2]. However, even when surgery can be performed, survival is still poor, with only 5-20% alive at 5 years [3]. The introduction of neoadjuvant chemotherapy and chemoradiotherapy has improved survival in several series, however there is still no consensus on whether neoadjuvant therapy is indicated in all operable cases [4]. When patients are not operable or they relapse after operation, chemotherapy can induce response rates in about 30-40%, however, survival of advanced esophageal cancer is poor, with a median survival of 7 to 8 months. Although there is no standard chemotherapy for advanced esophageal cancer, platinum based regimens have been used mostly in fit patients. The addition of new cytotoxic agents like paclitaxel and irinotecan seems to lead to higher response rates. A number of patients who progress after first line chemotherapy given for advanced disease, may still be fit for second line treatment. There is no drug presently available with substantial activity in this setting [4].

Gefitinib (ZD1839, IRESSA™) is a specific tyrosine kinase inhibitor (TKI) of EGFR [5] that has been approved in Japan and a number of other counties as single agent therapy for patients with refractory NSCLC. The response rate in Caucasian patients is 10-20%, while another 20-30% of the treated patients show stable disease for at least 2 months [6]. Somatic mutations within the EGFR kinase domain correlate with a dramatic clinical response to gefitinib in NSCLC patients [7,8]. Moreover, gefitinib-treated NSCLC patients with nuclear phospho-Akt tumor staining demonstrated an improved outcome [9]. Conversely, *k-ras* mutations are associated with primary resistance of NSCLC patients to EGFR-TKI therapy [10]. In contrast, EGFR, HER2, and phospho-Erk stainings did not predict for response of NSCLC patients to gefitinib [11,12].

The EGFR is a membrane-bound tyrosine kinase receptor that mediates growth and survival signals [13]. EGFR is activated upon binding of ligand to its extracellular domain, resulting in autophosphorylation and activation of downstream signaling molecules, such as Ras, Erk, PI3K, and Akt. The EGFR plays a prominent role in tumorigenesis, as it promotes growth of cells and is highly expressed and/or mutated in a variety of solid

tumor types, including esophageal cancer, NSCLC, and glioma [14]. EGFR overexpression was observed in 29-92% of esophageal tumors, which was correlated with poor patient prognosis and inferior response to therapy [15].

In this study we present the results of a phase II study of second-line gefitinib monotherapy for patients with advanced esophageal cancer, after failure of chemotherapy. We investigated the tumor material of these patients for a number of potential biological markers of activity.

Patients and Methods

This was a phase II study of gefitinib in patients who relapsed after chemotherapy for advanced esophageal cancer (1839IL/0059). The primary objectives of this study were to assess tumor response according to the RECIST criteria [16]; secondary endpoints were to estimate the duration of responses and progression-free survival, disease control rate and the tolerability of the treatment.

Patients

Entry criterias included: histologically confirmed carcinomas of the esophagus, WHO performance status of 2 or less, measurable or evaluable disease. Patients with cancers of the gastroesophageal junction were included if more than 50% of the tumor was localized in the esophagus. Patients had to have relapsed after one chemotherapy regimen, given at least 4 weeks before, and have white blood cells > 4000/mm^3 and platelets > 100000/mm^3, serum creatinine within 1.5 times the UNL (upper normal limit), and transaminases up to 5 times UNL in case of liver metastases. Patients had to be greater than 18 years old, and life expectancy was to be 12 weeks or longer. Excluded were patients that received prior treatment with an EGFR inhibitor, patients with brain metastases, those with unresolved toxicities from prior treatment, other active malignancies in the last 5 years, female patients who were pregnant or breast feeding, and patients using concomitant liver enzyme inducing medicines (e.g. phenobarbital, phenytoin).

Patients gave written informed consent and the study was approved by the ethical committees of the two institutions that participated in this study.

Treatment

Before enrolment patients underwent clinical examination, ECG, full blood counts, chemistries and urinalysis, and tumor assessment by CT scans of the chest and abdomen. During treatment, clinical examination, full blood counts, and chemistries were repeated every 2 weeks for the first 2 months, thereafter every month. Tumor assessment was repeated every 8 weeks. Tumor biopsies were performed at start and every 4 weeks whenever feasible. Gefitinib was given at 500 mg/day (two 250 mg tablets at each dosing) by oral route without interruption, unless severe toxicity ensued. In case of excessive toxicity interruptions were allowed up to 14 days. In case the side effect would not return to lower than grade 2, dose reduction to 250 mg/day was allowed. No more than one dose reduction was permitted.

Toxicity was assessed according to the NCI CTC grading system. Treatment continued until excessive toxicity, progression or patient request.

DNA isolation, PCR, and sequencing

Tumor specimens obtained at the time of primary diagnosis or study entry were collected to analyze the gene status of *EGFR*, *k-ras*, and *PIK3CA*. Paraffin-embedded tissue sections were macro-dissected and total genomic DNA was isolated using QIAamp DNA extraction Kits (Qiagen, Venlo, The Netherlands). Nested PCRs were carried out using the isolated DNA as template and external and internal primers as previously described [17]. Sequencing of PCR products was performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), M13 primers [17] and the ABI PRISM™ 310 Genetic analyzer (Applied Biosystems).

Immunohistochemical staining

Tumor slides were deparaffinized, autoclaved in 10 mM citrate buffer (pH 6.0), and incubated with EGFR antibody (VUMC), P-Akt (Ser 473) antibody (Cell Signaling, Beverly, MA; #9277), or P-p44/42 Mapk (Thr202/Tyr204) antibody (Cell Signaling; #9106) overnight at 4°C. The sections were developed using the DAKO EnVision™ visualization system. At present, there are no validated scoring systems for interpreting immununohistochemical staining for EGFR, P-Akt, or P-Erk. We used a system for interpreting EGFR staining that was based on scoring HER2 staining [18]: 0, none of the tumor cells stained; 1+, the staining of the tumor cell membranes was weak and incomplete; 2+, the staining of the tumor cell membranes was moderate and complete; 3+, the staining of the tumor cell membranes was strong and complete. Interpreting P-Akt and P-Erk staining was based on staining incidence and intensity: 0, none of the tumor cells stained; 1+, less than 10% of the tumor cells stained weakly; 2+, more than 10% of the tumor cells stained moderately; 3+, more than 25% of the tumor cells stained strongly. Interpretation of immunohistochemically stained slides was performed by a pathologist (G.M.) who was blinded to the clinical data.

CISH

Tumor sections were deparaffinized, boiled for 10 minutes in 1 mM EDTA/Tris (pH 9.0), digested with 0.01% pepsin/0.2 N HCl at 37°C, washed, and dried. Subsequently, the slides were incubated with the Zymed POT-light EGFR amplification probe (Zymed Laboratories Inc., San Francisco, CA) for 16-24h at 37°C after denaturation at 80°C. After blocking with goat anti-serum and incubation with a monoclonal anti-digoxin antibody (Clone DI-22; Sigma, Zwijndrecht, The Netherlands), the sections were developed using the DAKO EnVision™ visualization system. Similar to EGFR CISH analysis by Bhargava *et al.* [19], tumors were considered to be CISH+ when >50% of the tumor cells showed tight EGFR clusters or had >4 EGFR gene copies. Evaluation of the slides was performed independently by two authors, who were blinded to the patients' clinical characteristics and all other molecular variables.

Study design and statistical analysis

The study was performed as a phase II single agent trial, using a Fleming's single-stage design. The minimum required response rate of interest was set at 5% and the response of clinical interest was set at

20%. The required number of patients to be recruited was 38, based on a 90% power. Response rates and controlled disease rates were summarized by percentages and their 95% confidence intervals. Survival curves were constructed using the Kaplan-Meier method, and differences between the two groups were analyzed using the log-rank test. Patients groups were compared with the Spearman's Rho or Chi-square test when appropriate. All statistical analyses were performed using SPSS software (version 11.0.1). *, correlation is significant at the 0.05 level (2-tailed); **, correlation is significant at the 0.01 level (2-tailed).

Results

Patient characteristics and treatment outcome

Thirty-seven patients were screened and enrolled from February 2002 to February 2004, and 36 were eligible to the study and received at least one dose of gefitinib. One patient was found not to be eligible because of poor performance status and deteriorating conditions, and did not start treatment. The main patient characteristics are summarized in table 1. The majority of patients were male, with good performance status, adenocarcinoma and half of patients had prior surgery.

Of the 36 patients treated in the study, none had a complete response, one had a partial response (2.8%, 95% CI 0.1% - 14.5%), which lasted 3 months, 10 patients had stable disease (27.8%) and 17 patients progressed (47.2%). Eight patients (22.2%) were considered not evaluable for response, because the first evaluation was not performed due to early disease progression (n=2), adverse events (n=2), discontinuation of

Table 1. Patient characteristics.

Total number of eligible patients	36
Gender: male / female	25/11
Mean age (range), years	58 (36-75)
WHO performance status: 0 / 1 / 2	12/17/7
Histology	
Adenocarcinoma	26 (72.2%)
SCC	9 (25%)
Adenosquamous carcinoma	1 (2.8%)
Prior chemotherapy	
Platinum based	34 (94%)
5FU based	10 (28%)
Partial response	8 (22%)
Prior surgery	18 (50%)
Prior radiotherapy	12 (33%)

Table 2. Patient characteristics and association with response.

	PR/SD		PD/NE		
Characteristic	No.	(%)	No.	(%)	*P*
Sex					
Male	5	(45)	20	(80)	.038*
Female	6	(55)	5	(20)	
Age					
Median	58		59		
Range	38-69		36-75		
Histology					
ADC	5	(45.5)	21	(84)	.013*
SCC	5	(45.5)	4	(16)	
ADSC	1	(9)	0	(0)	
WHO performance	7	(64)	5	(20)	.160
0	1	(9)	16	(64)	
1	3	(27)	4	(16)	
2					
Prior surgery					
Yes	8	(73)	10	(40)	.070
No	3	(27)	15	(60)	
Prior radiotherapy					
Yes	2	(18)	10	(40)	.201
No	9	(82)	15	(60)	
Smoking[c]					
Never	4	(40)	10	(42)	.928
Former or Current	6	(60)	14	(58)	

[a] Response: PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable
[b] SCC, squamous cell carcinoma; ADC, adenocarcinoma; ADSC, adenosquamous carcinoma
[c] Smoking history is not known for two patients

gefitinib intake (n=2), or death before the first assessment (n=2). The disease control rate (response plus stable disease) was 30.6%. The median time to progression was 59 days (95% CI, 49 – 80 days). The proportion of patients alive and progression-free at 6 months was 18.5%. Of 3 patients still alive at the end of the study (6 months after the last inclusion), one patient was progression-free with a stable disease and on treatment. Median survival time was 164 days (95% CI= 0 to 333 days) and 6 patients (16.7%) were alive at one year (Fig. 1). Interestingly, controlled disease was significantly associated with being female and SCC histology (Table 2).

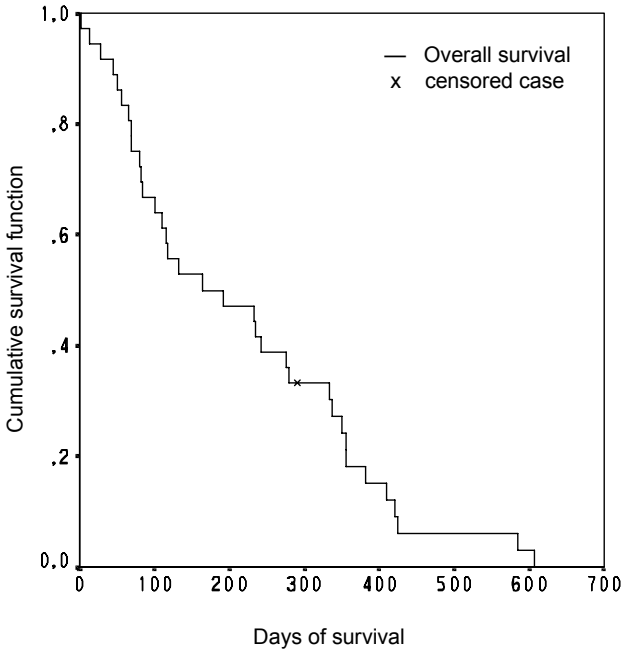


Figure 1. Kaplan-Meier curve for overall survival of pretreated esophageal cancer patients treated with gefitinib. Survival was calculated from the date of gefitinib therapy initiation to the date of death. Median survival time was 164 days (95% CI= 0 to 333 days).

Side effects

The major reason for treatment discontinuation was disease progression in 29 patients (80.6%). Five patients had treatment interruption due to toxicity and 4 patients had a dose reduction. The most common gefitinib related side effect was diarrhea (58.3%), followed by rash (47.2%). Main side effects are reported in table 3. Severe side effects were relatively infrequent, and in general the treatment was well tolerated. No toxic death was reported.

Table 3. Most common side effects to gefitinib (n=36)

Toxicity	All grades (%)	Grade 3-4 (%)
Diarrhea	21 (58.3)	3 (8.3)
Rash	17 (47.2)	1 (2.8)
Dry skin	4 (11.1)	
Erythema	4 (11.1)	
Increased AST	5 (13.9)	1 (2.8)
Increased ALT	3 (8.3)	1 (2.8)
Vomiting	3 (8.3)	1 (2.8)
Nausea	4 (11.1)	
Stomatitis	4 (11.1)	

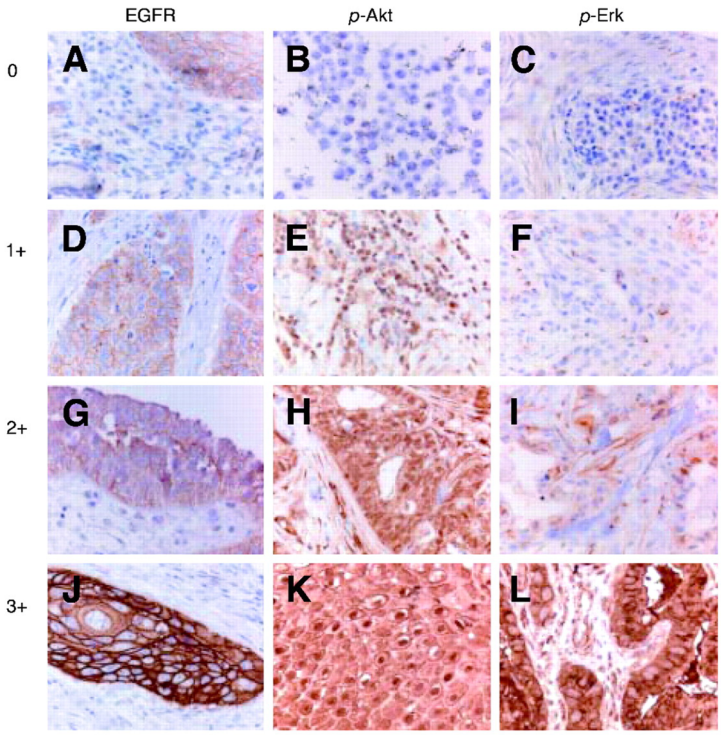


Figure 2. EGFR, p-Akt, and p-Erk staining as determined by immunohistochemistry. Panels show representative examples of esophageal tumor specimens stained for total EGFR (A, D, G, J), phosphorylated (p) Akt (B, E, H, K), and phosphorylated (p) Erk (C, F, I, L). Panels illustrate specimens with 0 (A-C), 1+ (D-F), 2+ (G-I), and 3+ (J-L) expression levels. All esophageal tumors were positive for p-Akt, hence SW1573 NSCLC cells were shown as a control for negative staining (0) of p-Akt (B).

EGFR, k-ras *and* PIK3CA *mutational analysis*

Esophageal tumors were evaluated for the presence of activating mutations in exons 18 to 21 of the *EGFR* gene [7,8]. All of the 26 evaluated esophageal tumors were wild type for this region of the *EGFR* gene. Esophageal tumors were additionally screened for activating mutations in the *k-ras* and *PIK3CA* genes, which may confer resistance to gefitinib. Although no mutations were found in hotspot regions of the *PIK3CA* gene in 24 tumors tested, two out of 23 evaluated tumors (8.7 %) harbored point mutations in codon 12 or 13 of the *k-ras* gene (data not shown). Both patients with a *k-ras* mutation had (early) disease progression upon gefitinib treatment.

Expression of EGFR

Among the 24 tumors evaluated for EGFR expression, 15 (62.5%) had low or moderate (0/1+/2+) EGFR expression and 9 (37.5%) were highly positive (3+) for EGFR (Table 4; Fig. 2). High EGFR expression was significantly associated with SCC histology, but not with other clinical parameters (Table 4). The disease control rate was 66.7% for patients with high EGFR expressing tumors, which was significantly higher than for patients with low to moderate EGFR expressing tumors (Table 5). The time to

progression was longer for patients with high EGFR expressing tumors (median 153 days, 95% CI 36-270 days) than for patients with low or moderate EGFR expressing tumor (median 55 days, 95% CI 42-68 days), although this was not statistically significant (Fig. 3A). The overall survival for patients with high EGFR expressing tumors (median 233 days, 95% CI 110-356 days) was longer compared to that of patients with low or moderate EGFR expressing tumors (median 83 days, 95% CI 25-141 days), but this was not significantly different (Fig. 3B).

Table 4. Patient characteristics and association with EGFR, p-Akt, and p-Erk status determined by immunohistochemistry.

	EGFR status				p-Akt status				p-Erk status			
	Total	EGFR 0/1+/2+ (%)	EGFR 3+ (%)		Total	p-Akt 1+/2+ (%)	p-Akt 3+ (%)		Total	p-Erk 0/1+ (%)	p-Erk 2+/3+ (%)	
Characteristic	n=24	15 (62.5)	9 (37.5)	*P*	n=13	6 (46.2)	7 (53.8)	*P*	n=22	11 (50)	11 (50)	*P*
Sex												
Male	18 (75)	12 (80)	6 (66.7)	.465	10 (76.9)	3 (50)	7 (100)	.033*	16 (72.7)	8 (72.7)	8 (72.7)	.856
Female	6 (25)	3 (20)	3 (33.3)		3 (23.1)	3 (50)	0 (0)		6 (27.3)	3 (27.3)	3 (27.3)	
Age												
Median	59	58	59		59	65	58		59	57	64	
Range	38-69	51-67	38-69		51-69	55-69	51-67		38-69	38-69	51-67	
Histology												
ADC	18 (75)	14 (93.3)	4 (44.4)	.007**	8 (61.5)	2 (33.3)	6 (85.7)	.053	16 (72.7)	5 (45.5)	11	.030*
SCC	6 (25)	1 (6.7)	5 (55.6)		5 (38.5)	4 (66.7)	1 (14.3)		6 (27.8)	6 (54.5)	(100)	
											0 (0)	
WHO performance	7 (29.2)	3 (20)	4 (44.4)	.542	4 (30.8)	2 (33.3)	2 (28.6)	.550	6 (27.3)	2 (18.2)	4 (36.4)	.067
0	13 (54.2)	10 (66.7)	3 (33.3)		7 (53.8)	2 (33.3)	5 (71.4)		12 (54.5)	5 (45.5)	7 (63.6)	
1	4 (16.7)	2 (13.3)	2 (22.2)		2 (15.4)	2 (33.3)	0 (0)		4 (18.2)	4 (36.4)	0 (0)	
2												
Prior surgery												
Yes	11 (45.8)	6 (40)	5 (55.6)	.459	8 (61.5)	5 (83.3)	3 (42.9)	.135	10 (45.5)	7 (63.6)	3 (27.3)	.571
No	13 (54.2)	9 (60)	4 (44.4)		5 (38.5)	1 (16.7)	4 (57.1)		12 (54.5)	4 (36.4)	8 (72.7)	
Prior radiotherapy	9 (37.5)	6 (40)	3 (33.3)	.744	4 (30.8)	2 (33.3)	2 (28.6)	.853	7 (31.8)	6 (54.5)	1 (9.1)	.015*
Yes	15 (62.5)	9 (60)	6 (66.7)		9 (69.2)	4 (66.7)	5 (71.4)		15 (68.2)	5 (45.5)	10	
No											(90.9)	

Expression of p-Akt

Stainings of esophageal tumor sections for p-Akt were difficult to interpret, as particularly adenocarcinomas showed p-Akt staining (1+ to 2+) of surrounding normal tissue. However, tumor tissue usually showed strong, nuclear p-Akt staining compared to weaker staining of surrounding normal tissue. When considering tissues with tumor-specific staining only, 13 tumors were evaluable. Of these tumors, 7 were highly positive (3+) for p-Akt, and 6 expressed low to moderate (1+/2+) expression of p-Akt (Table 4). High p-Akt staining was associated with being male, but not with other patient characteristics (Table 4). Patients with low p-Akt expression had better disease control (Table 5) and longer progression free survival compared to patients with high p-Akt

status (median 153 days, 95% CI: 0-316 days vs. median 49 days, 95% CI: 37-61 days) (Fig. 3C). However, overall median survival was not significantly different on the basis of p-Akt status (191 days, 95% CI: 0-388 days vs. 133 days, 95% CI: 87-179 days) (Fig. 3D).

Expression of p-Erk

Of the 22 tumors that were stained for p-Erk, 14 (63.6%) had negative (0/1+) p-Erk expression and 8 tumors (36.4%) showed positive (2+/3+) p-Erk expression (Table 3; Figure 3E, F). High Erk phosphorylation was observed more frequently in adenocarcinomas and in patients that had no prior radiotherapy (Table 3). Disease control rates, the median time to progression (56 days, 95% CI: 41-70 days vs. 62 days, 95% CI: 27-97 days), and the median survival time (115 days, 95% CI: 82-148 days vs. 234 days, 95% CI: 0-535 days) were similar among patients whose tumors differed on the basis of p-Erk status (Table 4, Fig. 3E, F).

EGFR gene copy number

EGFR gene copy numbers of 16 tumors were evaluated by CISH. Two tumors (12.5%) were considered CISH+ (data not shown), among which the tumor from the patient with a partial response and a patient with disease progression upon gefitinib treatment. Similar to recent findings of Hanawa *et al.* [20], the two CISH+ cases showed high (3+) EGFR protein expression and SCC histology.

Table 5. Response of gefitinib therapy in patients with advanced esophageal cancer according to total EGFR, phosphorylated Akt, and phosphorylated Erk expression.

Tumor status		Total No. of evaluated patients (%)	No. of partial responses and stable disease (%)	*P*
Total		36	11 (30.6)	
EGFR	Total	24	7 (29.2)	
	0/1+/2+	15	1 (6.7)	.002**
	3+	9	6 (66.7)	
p-Akt	Total	13	4 (30.7)	
	1+/2+	6	4 (66.7)	.009**
	3+	7	0 (0)	
p-Erk	Total	22	7 (31.8)	
	0/1+	11	4 (36.4)	.604
	2+/3+	11	3 (27.3)	

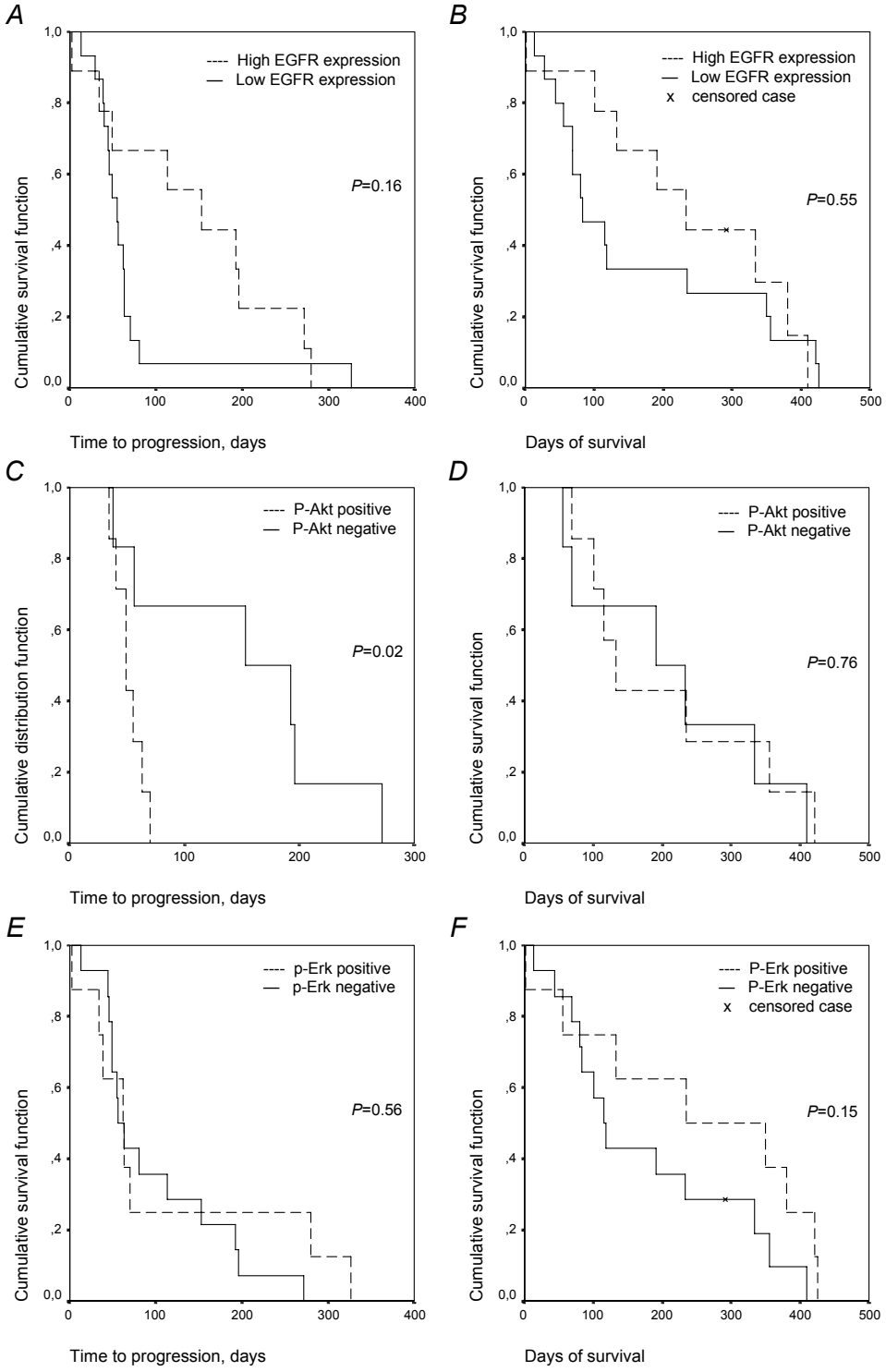


Figure 3. Kaplan-Meier curves for time to disease progression (A, C, E) and survival (B, D, F) according to protein expression levels of EGFR (A-B), phosphorylated (p-) Akt (C-D), and phosphorylated (p-) Erk (E-F). Time to disease progression was calculated from the date of initiation of gefitinib treatment to the date of detection of progressive disease. Survival was calculated from the date of therapy initiation to the date of death. Statistical significance of differences between groups was evaluated with the log-rank test.

Discussion

Gefitinib given at 500 mg/day in second-line treatment of advanced esophageal cancer, failing after chemotherapy induced one partial response. Although this response rate (2.8%) is disappointing, 30.6% of patients demonstrated controlled disease. These results suggest that gefitinib as monotherapy is less effective than historical controls which, with cytotoxic chemotherapy regimes have recorded response rates of 12-30% for patients who have received 2nd line therapy, with progression free survivals of approximately 3.5 months [21,22] in comparison to a progression free survival of 2 months in this study. The safety profile of gefitinib given at 500 mg is similar to other studies with this agent and demonstrates that the drug is well tolerated. The overall adverse events profile was, as expected, predominated by gastro-intestinal and skin related events.

Although the clinical results were disappointing, we attempted to identify the patients that had benefit from gefitinib treatment, in particular the patients who had a response or stable disease (Table 6).

Table 6. Characteristics of non-progressive esophageal cancer patients upon gefitinib therapy.

Patient no.	Response[a]	Sex	Age	Histology[b]	WHO performance status	Prior surgery	Prior radiotherapy	Mutational analysis[c]			Immunohistochemistry[d]		
								EGFR	*PIK3CA*	*k-Ras*	EGFR status	p-Akt status	p-Erk status
0601.04	SD	M	68	SCC	2	Yes	Yes	WT	WT	WT	3+	2+	0
0601.05	SD	M	52	ADC	1	Yes	No	WT	WT	WT	3+	NE	3+
0601.15	SD	M	63	ADC	2	Yes	No	WT	WT	WT	NE	NE	NE
0601.21	SD	F	58	ADC	0	Yes	No	WT	WT	WT	2+	NE	3+
0613.01	SD	F	58	SCC	0	Yes	No	WT	NE	NE	3+	1+	1+
0613.02	PR	F	69	SCC	0	Yes	No	WT	WT	WT	3+	2+	0
0613.05	SD	F	51	ADC	0	No	No	NE	NE	NE	NE	NE	NE
0613.07	SD	M	60	SCC	0	No	No	NE	NE	NE	NE	NE	NE
0613.08	SD	F	62	SCC	2	Yes	Yes	WT	WT	WT	3+	2+	0
0613.09	SD	F	52	ADSC	0	Yes	No	NE	NE	NE	NE	NE	NE
0613.10	SD	M	38	ADC	0	No	No	WT	WT	WT	3+	NE	3+

[a] Response: PR, partial response; SD, stable disease
[b] SCC, SCC; ADC, adenocarcinoma; ADSC, adenosquamous carcinoma
[c] *EGFR*, *PIK3CA*, and *k-Ras* mutational status was determined by direct DNA sequencing. WT, wild type.
[d] EGFR and phosphorylation status of Akt and Erk were determined by immunohistochemistry. For definition of degree of expression see materials and methods. NE, Not Evaluable

Analogous to studies evaluating gefitinib in NSCLC [6], being female predicted for a better patient outcome in this study. In contrast to lung cancer studies, we found that SCC histology was significantly associated with a better patient outcome. Our results are consistent with a large phase II trial using gefitinib in SCCs of the head and neck

(n=47), in which a response rate of 10.4 % was reported, while 53% of patients experienced stable disease [23]. These numbers are similar to the response and stable disease rates of 12.5% and 50%, respectively, within the group of patients with SCC histology (n=8) in the study presented here. Therefore, the fact that the majority of the patients were male and had adenocarcinoma histology may have decreased the overall response rate in this study. Thus, selection of patients on the basis of SCC histology might have improved the outcome.

In addition to clinical parameters, we investigated several potential biological markers of gefitinib activity. Given the fact that only a part of the tumors were evaluable for biomarker-analysis, the results presented in this study are unfortunately not conclusive. The finding that *EGFR* kinase domain mutations were not detected in esophageal tumors may be due to the low frequency of *EGFR* mutations detected in tumors other than NSCLC [24]. In line with our results, no *EGFR* mutations were reported in a recent study evaluating 40 esophageal SCCs [20]. On the other hand, we did not observe such dramatic clinical responses upon treatment with gefitinib in this study as those that have been described for *EGFR* mutant NSCLC patients treated with EGFR-TKIs [7,8]. However, patients with high EGFR expression experienced significantly better-controlled disease and demonstrated a trend towards improved time to progression and overall survival while on treatment. Our data suggest that patients with high EGFR expression may have a better prognosis when treated with EGFR-TKIs, as high EGFR expression has been shown to be a bad prognostic factor for esophageal cancer patients [25,26]. As esophageal cancers overexpress EGFR in 29-92% of cases [15], it is conceivable that a higher response or disease control rate might be achieved if patients were selected on the basis of high EGFR expression.

Two out of four cases with high EGFR expression were also CISH+ (data not shown). This suggests that EGFR overexpression is caused by increased EGFR gene copy number in just a fraction of esophageal tumors, like Hanawa *et al.* reported recently [20]. Our data further suggest that analysis of EGFR expression by immunohistochemistry is more effective to predict gefitinib efficacy in esophageal cancer patients than EGFR gene copy number, in contrast to NSCLC [11,12,27]. However, direct comparisons are difficult between the immunohistochemical data of the different studies because of differences in antibodies used and interpretation of the results. The introduction of a standardized system for scoring EGFR immunohistochemical stainings is needed.

EGFR-independent activity of the downstream Ras/Erk and PI3K/Akt pathways confers resistance of tumor cells to gefitinib treatment [28]. Similar to NSCLCs [10,17], the presence of *k-ras* mutations in esophageal tumors may be a factor of resistance to gefitinib, as two patients with *k-ras* mutant tumors were progressive upon gefitinib therapy. However, phosphorylation of Erk, downstream of k-ras [29], was not elevated in tumors with *k-ras* mutations and p-Erk status was not predictive for outcome to gefitinib treatment. No mutations were detected in the *PIK3CA* gene in 24 esophageal tumors. *PIK3CA* mutations are relatively frequent in colorectal, breast and ovarian tumors, but less frequent in other tumor types [30,31]. Low p-Akt staining is associated with better disease control and longer progression-free survival in the small group of patients evaluated. However, p-Akt status, downstream of PI3K [32], will not be a good predictive factor for gefitinib therapy in esophageal cancer, because of the difficult evaluation of phosphorylated Akt levels.

In conclusion, gefitinib treatment of unselected patients with advanced, pretreated esophageal cancer has modest activity. However, being female, high EGFR expression levels, and SCC histology are associated with better patient outcome. The selection of esophageal cancer patients for future studies with EGFR-TKIs based on the level of EGFR expression in their tumors or SCC histology should be considered.

References

1. American Cancer Society. What are the key statistics about cancer of the esophagus? Revised: 03/09/2005. http://www.cancer.org/docroot/CRI/content/CRI_2_4_1X_What_are_the_key_statistics_for_esophagus_cancer_12.asp?sitearea= (2005).

2. Hartel, M *et al.* Surgical treatment of oesophageal cancer. *Dig Dis* **22**, 213-220 (2004).

3. Urba, SG *et al.* Gemcitabine and cisplatin for patients with metastatic or recurrent esophageal carcinoma: a Southwest Oncology Group Study. *Invest New Drugs* **22**, 91-97 (2004).

4. Enzinger, PC *et al.* Esophageal cancer. *N Engl J Med* **349**, 2241-2252 (2003).

5. Wakeling, AE *et al.* ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. *Cancer Res* **62**, 5749-5754 (2002).

6. Fukuoka, M *et al.* Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. *J Clin Oncol* **21**, 2237-2246 (2003).

7. Paez, JG *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304**, 1497-1500 (2004).

8. Lynch, TJ *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* **%20;350**, 2129-2139 (2004).

9. Cappuzzo, F *et al.* Akt phosphorylation and gefitinib efficacy in patients with advanced non-small-cell lung cancer. *J Natl Cancer Inst* **96**, 1133-1141 (2004).

10. Pao, W *et al.* KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med* **2**, e17 (2005).

11. Cappuzzo, F *et al.* Gefitinib in pretreated non-small-cell lung cancer (NSCLC): analysis of efficacy and correlation with HER2 and epidermal growth factor receptor expression in locally advanced or metastatic NSCLC. *J Clin Oncol* **21**, 2658-2663 (2003).

12. Parra, HS *et al.* Analysis of epidermal growth factor receptor expression as a predictive factor for response to gefitinib ('Iressa', ZD1839) in non-small-cell lung cancer. *Br J Cancer* **%19;91**, 208-212 (2004).

13. Yarden, Y *et al.* Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2**, 127-137 (2001).

14. Salomon, DS *et al.* Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* **19**, 183-232 (1995).

15. Kuwano, H *et al.* Genetic alterations in esophageal cancer. *Surg Today* **35**, 7-18 (2005).

16. Therasse, P *et al.* New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* **92**, 205-216 (2000).

17. Janmaat, ML *et al.* Enhanced cytotoxicity induced by gefitinib and specific inhibitors of the Ras or phosphatidyl inositol-3 kinase pathways in non-small cell lung cancer cells. *Int J Cancer* **118**, 209-214 (2006).

18. Hatanaka, Y *et al.* Quantitative immunohistochemical evaluation of HER2/neu expression with HercepTestTM in breast carcinoma by image analysis. *Pathol Int* **51**, 33-36 (2001).

19. Bhargava, R *et al.* EGFR gene amplification in breast cancer: correlation with epidermal growth factor receptor mRNA and protein expression and HER-2 status and absence of EGFR-activating mutations. *Mod Pathol* **18**, 1027-1033 (2005).

20. Hanawa, M *et al.* EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. *Int J Cancer* **.** (2005).

21. Assersohn, L *et al.* Phase II study of irinotecan and 5-fluorouracil/leucovorin in patients with primary refractory or relapsed advanced oesophageal and gastric carcinoma. *Ann Oncol* **15**, 64-69 (2004).

22. Kelsen, DP. Multimodality therapy of esophageal cancer: an update. *Cancer J* **6 Suppl 2:S177-81.**, S177-S181 (2000).

23. Cohen, EE *et al.* Phase II trial of ZD1839 in recurrent or metastatic squamous cell carcinoma of the head and neck. *J Clin Oncol* **21**, 1980-1987 (2003).

24. Pao, W *et al.* Epidermal growth factor receptor mutations, small-molecule kinase inhibitors, and non-small-cell lung cancer: current knowledge and future directions. *J Clin Oncol* **23**, 2556-2568 (2005).

25. Wilkinson, NW *et al.* Epidermal growth factor receptor expression correlates with histologic grade in resected esophageal adenocarcinoma. *J Gastrointest Surg* **8**, 448-453 (2004).

26. Yacoub, L *et al.* Transforming growth factor-alpha, epidermal growth factor receptor, and MiB-1 expression in Barrett's-associated neoplasia: correlation with prognosis. *Mod Pathol* **10**, 105-112 (1997).

27. Cappuzzo, F *et al.* Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. *J Natl Cancer Inst* **97**, 643-655 (2005).

28. Janmaat, ML *et al.* Response to epidermal growth factor receptor inhibitors in non-small cell lung cancer cells: limited antiproliferative effects and absence of apoptosis associated with persistent activity of extracellular signal-regulated kinase or Akt kinase pathways. *Clin Cancer Res* **9**, 2316-2326 (2003).

29. Bos, JL. p21ras: an oncoprotein functioning in growth factor-induced signal transduction. *Eur J Cancer* **31A**, 1051-1054 (1995).

30. Campbell, IG *et al.* Mutation of the PIK3CA gene in ovarian and breast cancer. *Cancer Res* **64**, 7678-7681 (2004).

31. Samuels, Y *et al.* High frequency of mutations of the PIK3CA gene in human cancers. *Science* **304**, 554 (2004).

32. Burgering, BM *et al.* Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599-602 (1995).

Chapter 5

Kahalalide F induces necrosis-like cell death that involves depletion of ErbB3 and inhibition of Akt signaling

Maarten L Janmaat
José A Rodriguez
José Jimeno
Frank AE Kruyt
Giuseppe Giaccone

Molecular Pharmacology 2005, 68:502-10

Abstract

Kahalalide F (KF) is a novel marine-derived antitumor agent that is currently undergoing phase II clinical trials. The mechanism of action of KF is not well understood. In line with previous reports, we show that KF caused rapid and potent cytotoxicity in the breast cancer cell lines SKBR3 and BT474, characterized by cytoplasmic swelling and DNA clumping. Several markers of caspase-dependent apoptosis, such as phosphatidyl-serine externalization, cytochrome C release, and caspase-3 and PARP cleavage were negative after KF exposure. Inhibitors of caspases or cathepsins failed to protect against KF-cytotoxicity. Altogether, these data indicate that KF-induced cell death is a necrosis-like process. The sensitivity to KF in a panel of human tumor cell lines derived from breast (SKBR3, BT474, MCF7), vulval (A431), non-small cell lung (H460, A549, SW1573, H292), and hepatic (Skhep1, HepG2, Hep3B) carcinoma, positively correlated with ErbB3 (HER3) protein levels. A KF-resistant subline of colon carcinoma cells, HT29/KF, expressed significantly reduced levels of all ErbB receptors, but short term KF exposure of sensitive cell lines such as SKBR3 selectively induced downregulation of ErbB3. Conversely, stable transfection of an ErbB3-expressing plasmid increased the KF sensitivity of H460 cells, the most resistant cell line in our panel. Finally, we found that KF efficiently inhibited the PI3K-Akt signaling pathway in sensitive cell lines and that ectopic expression of a constitutively active Akt mutant reduced KF cytotoxicity in this cell line. In summary, our results identify ErbB3 and the downstream PI3K-Akt pathway as important determinants of the cytotoxic activity of KF *in vitro*.

Introduction

Kahalalide F (KF) is a novel antitumor agent that was originally isolated from the Hawaiian marine mollusk *Elysia rufescens* [1,2]. KF has high cytotoxic activity against cell lines and tumor specimens derived from various human solid tumors, including prostate, breast, NSCL, ovarian, and colon carcinomas [3-7]. Moreover, KF has shown antitumor activity against human prostate cancer xenografts in mouse models [3]. In contrast, non-tumoral cell lines were 5-40 times less sensitive to KF [8] and bone marrow progenitors were not affected when treated with supra-pharmacological concentrations of KF [9]. In a phase I clinical trial in solid tumors antitumor activity has been noted in patients harboring hepatoma, melanoma, and breast and pancreatic carcinoma [10]. Currently, the activity of KF is being investigated in phase II clinical trials in patients with melanoma, hepatic carcinoma, an NSCLC [11].

The molecular mechanism of action of KF is largely unknown. Lysosomes appear to be an intracellular target of KF, as human cervical tumor cells and monkey fibroblasts treated with KF formed large vacuoles and became dramatically swollen due to changes in lysosomal membranes [12]. Consistently, a recent report demonstrated the loss of lysosomal integrity and the induction of severe cytoplasmic swelling and vacuolization in breast and prostate cancer cells [13]. In the latter study, confocal laser and electron microscopy revealed that KF also induces damage of mitochondria, endoplasmatic reticulum, and the plasma membrane. In contrast, the nuclear membrane was preserved and no DNA damage was detected, although the cell nucleus showed irregular clumping of chromatin. Ectopic overexpression of the multidrug resistance protein MDR1, inhibition of protein synthesis, or inhibition of caspase-dependent apoptosis did not significantly protect against KF cytotoxicity [14].

Inhibition of ErbB signaling has been suggested to be part of the mechanism of KF action [4,15], although ectopic overexpression of ErbB2 did not protect against KF-induced cell death [16]. Here we show that KF-induced cytotoxicity does not involve caspase-mediated apoptosis, but is a necrosis-like cell death process. KF-induced cell death was also independent from the activity of the lysosomal proteases cathepsin B and D. The sensitivity to KF in a panel of cell lines derived from several tumors types, including breast, vulval, NSCL, and

hepatic carcinoma, significantly correlated with protein expression levels of the ErbB3 receptor. We show that KF exposure induced downregulation of ErbB3, while ectopic expression of ErbB3 increased the KF sensitivity of a resistant cell line. Finally, we found that KF efficiently inhibited the PI3K-Akt signaling pathway in sensitive cell lines, and that ectopic expression of a constitutively active Akt mutant reduced KF cytotoxicity. Altogether, our data identify ErbB3 and Akt as major determinants of the cytotoxic activity of KF *in vitro*.

Materials and Methods

Chemicals

KF (1-Oxa-4,7,10,13,16-pentaazacyclononadecane, cyclic peptide derivative) is manufactured by Pharmamar (Madrid, Spain) and was provided as a pure substance and diluted in dimethyl sulfoxide (DMSO): ethanol (1:1; v/v). Stock solutions of zVAD-fmk (Enzyme Systems Products, Livermore, CA), calpeptin (Calbiochem, Darmstadt, Germany), CA-074 Me (Peptides International, Osaka, Japan), zFA-fmk (Enzyme Systems Products), pepstatin A (Sigma Chemicals, St. Louis, MO), and LY294002 (Cell Signaling Technology) were made in DMSO.

Cell culture and KF selection

SKBR3, BT474, MCF7 (breast carcinoma), A431 (vulval carcinoma), NCI-H460 (H460), A549, SW1573, NCI-H292 (H292) (NSCLC), SKhep1, HepG2, Hep3B (hepatic carcinoma), and HT29 (colon carcinoma) cell lines were grown at 37° C and 5% CO_2 in Dulbecco's modified Eagle's medium or RPMI-1640 (BioWhittaker) supplemented with 10% (v/v) fetal calf serum (Life Technologies, Breda, The Netherlands), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco BRL). Cells from exponentially growing cultures were used in all experiments. The KF-resistant HT29 cell line (HT29/KF) (supplied by Dr. Lola Garcia Grávalos, Pharmamar, Madrid, Spain) was obtained by treating parental HT29 cells with increasing concentrations of KF, from 0.15 µM KF to 1.3 µM KF, in which the cells grew well after 20 weeks of selection.

Flow cytometry

Cells were plated at 40-60% confluency in 6- or 12-well plates. The following day, the medium was replaced with medium containing the drug(s), as indicated. Cells were treated for various times with 1 µM KF, unless otherwise indicated. Protease inhibitors were diluted in medium at final concentrations of 50 µM (zVAD-fmk), 100 µM (calpeptin, CA-074 Me, pepstatin A) or 200 µM (zFA-fmk), and added to the cells 1 hr prior to addition of KF. Cell cycle analysis and apoptosis measurement were performed as previously described [17]. Briefly, the cell cycle distribution was determined by propidium iodide (PI) staining of cells, which were resuspended in Nicoletti buffer [18] and analyzed by flow cytometry. The extent of cell death was determined by

the analysis of hypodiploid DNA using PI-staining, or by annexin V-FITC and 7-amino-actinomycin D (7-AAD) double staining according to the manufacturer's protocol (Nexins Research, Kattendijk, The Netherlands). All analyses were performed on a FACScalibur instrument using the CellQuest software package (Becton Dickinson, Mount View, CA).

Western Blotting

Whole cell lysates were denatured in sample buffer containing SDS and equal amounts of total protein were separated on 8-15 % SDS-poly-acrylamide gels and transferred to nitrocellulose membranes. After blocking with 5 % nonfat dry milk, the membranes were incubated O/N at 4° C with the first antibodies as indicated. The next day, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies, and detection was performed using enhanced chemiluminescence reagent (Amersham). The antibodies used are: anti-caspase-3, anti-phospho-EGFR (Tyr1068), anti-Erk, anti-phospho-Erk, anti-Akt, anti-phospho-Akt (Ser473), anti-phospho-MDM2, anti-phospho-GSK-3β (all from Cell Signaling Technology), anti-PARP (Roche), anti-EGFR (Ab-12, Neomarkers), anti-c-ErbB2 (C18, Santa Cruz), anti-phospho-c-ErbB2 (Tyr1248; Neomarkers), anti-c-ErbB3 (Ab-2, Neomarkers), anti-c-ErbB-4 (Ab-2, Neomarkers). Protein expression levels were quantified using Biorad software and normalized with β-actin expression levels.

MTT assays

5 x 10^3 cells were plated into flat bottom 96-well plates (Costar, Corning, NY). After 24 h, various concentrations of KF were added and the cells were incubated for an additional 72 h. Subsequently, 10% (v/v) of a solution of 5 mg/ml 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added to each well and incubated for 3 h at 37° C. Plates were centrifuged for 5 min at 1200 rpm and the medium was carefully discarded. The formed formazan crystals were dissolved in 100 μl DMSO and absorbance was determined at 540 nm using a Spectra Fluorimeter (Tecan, Salzburg, Austria). Absorbance values were expressed as the percentage of the untreated controls to calculate the concentration of KF resulting in 50% growth inhibition (IC_{50}).

Immunocytochemistry, fluorescence microscopy analysis and quantification of subcellular distribution

Cells growing onto glass coverslips were fixed with 3.7% formaldehyde in PBS for 30 min, washed with PBS, permeabilized with 0.2% Triton in PBS for 10 min, and washed with PBS again. Following a blocking step with 3% bovine serum albumin in PBS for 1 h, primary antibodies against $p27^{kip1}$ (BD Biosciences) or cytochrome c were diluted in blocking solution and applied for 1 h. After washing with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-(Sigma) or Alexafluor Red- (Molecular Probes, Eugene, OR) conjugated secondary antibodies for 1 h. Finally, the coverslips were rinsed three times with PBS and mounted onto microscope slides with Vectashield (Vector). The chromosome dye Hoechst 33285 (Sigma) was used to

counterstain the nuclei. The immunostaining procedure was carried out at room temperature. Fluorescence microscopy analysis was carried out using an inverted Leica DMIRB/E fluorescence microscope (Leica Heidelberg, Heidelberg, Germany). Images were collected using the Q500MC Quantimet software V01.01 (Leica Cambridge, Cambridge, U.K.). To quantify the subcellular distribution of $p27^{kip1}$, the localization of the protein was determined in at least 200 cells per treatment.

Transfections

The mammalian expression vectors pBABE-ErbB3 [19] and pSG5-gagPKB [20] were generously provided by Dr. N. Hynes (Friedrich Miescher Institute, Basel, Switzerland) and Dr. P. Coffer (University Medical Center, Utrecht, The Netherlands), respectively. Cells were seeded in 6- or 12-well trays, and transfected with 0.5-2 µg of plasmid DNA using the FuGene6 transfection reagent (Roche Molecular Biochemicals), according to the manufacturer's protocol. For transient transfection experiments, cells were transfected with the YFP-expression plasmid pEYFP-C1 (Clontech, Palo Alto, CA, U.S.A.) alone or together with the pSG5-gagPKB vector. Using YFP expression as a marker of transfection, we determined the percentage of transfected cells that detached after treatment with KF. Expression of gag-PKB was confirmed by Western blotting in a parallel sample. Finally, to make stable ErbB3 expressing cells, H460 cells were transfected with 5-10 µg pBABE-ErbB3 cDNA or empty vector control vectors, using Superfect reagent (Gibco BRL) according to the manufacturer's protocol. Transfected cells were selected in puromycin-containing medium and the pooled population was used.

Statistics

Correlation coefficients (*r*) and accompanying *P*-values were calculated using SPSS software. Quantitative experiments were analyzed by the Student's *t*-test and *p*-values resulted from the use of two-sided tests and were considered significant when ≤ 0.05.

Results

KF-induced cytotoxicity is mediated through a necrosis-like cell death process

To investigate if KF induces the activation of caspase-dependent apoptosis, we examined several apoptotic markers after treatment with KF. First, cell cycle analysis revealed that KF induces a hypodiploid cell population in SKBR3 and BT474 cells, starting after 16 to 24 h exposure to KF and increasing to almost 100% after 72 h (Figure 1A). Flow cytometric analysis after 7-AAD/Annexin-V double staining demonstrated that KF caused a rapid disruption of the cell membrane, as visualized by the increase of 7-AAD staining (Figure 1B). However,

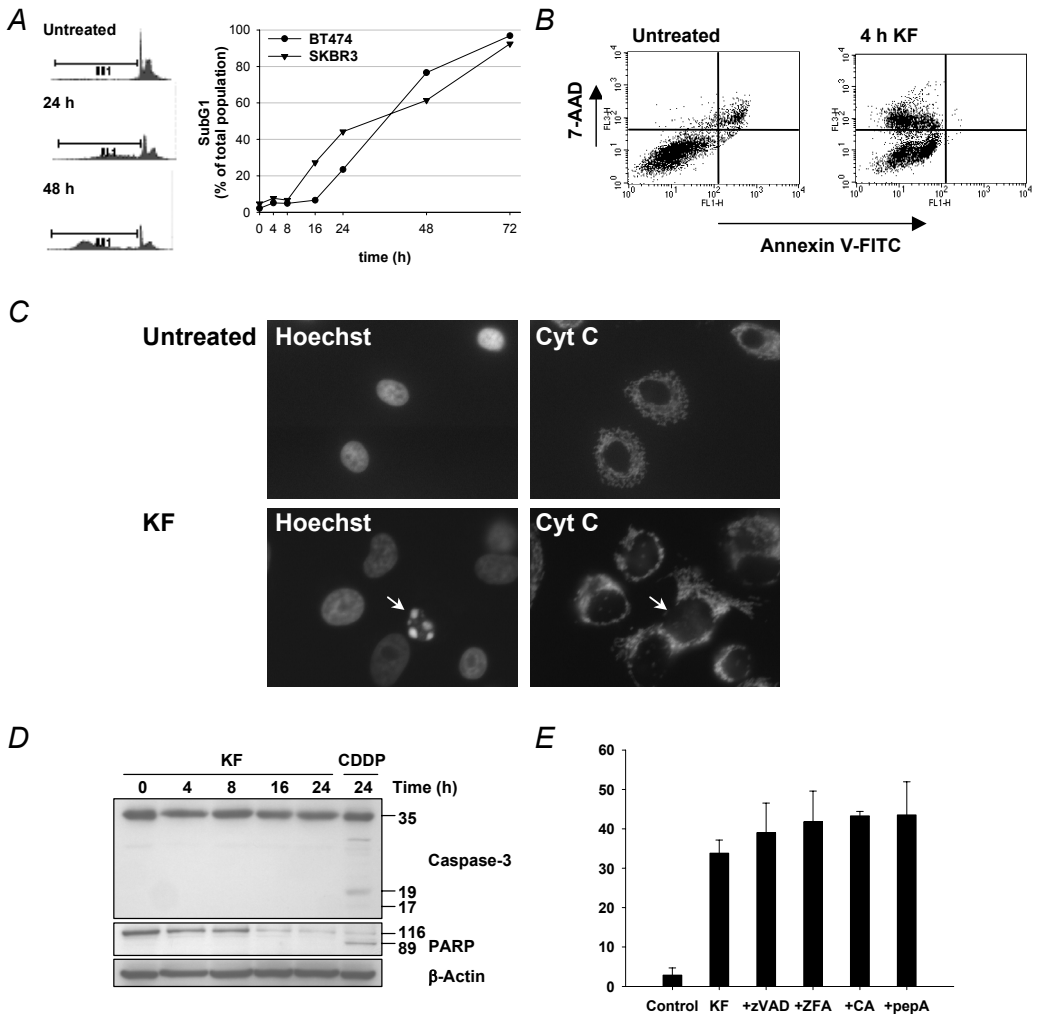


Figure 1. KF induces necrosis-like cell death. *A,* flow cytometry analysis of the DNA content of BT474 and SKBR3 cells stained with propidium iodide (PI) after exposure to KF (1 μM) for different times. Representative examples of the cell cycle distribution of SKBR3 cells untreated or treated for 24 or 48 h with KF are shown (left panels). The amount of cells with hypodiploid DNA content (M1) was measured and presented as percentage of total cell population (right panel). *B,* KF-treated SKBR3 cells (1 μM for 4h) were double-stained with annexin-V/7-AAD and analyzed by flow cytometry. Gate-settings distinguish between living (lower left), necrotic (upper left), early apoptotic (lower right) and late apoptotic (upper right) cells. *C,* Cytochrome C is not released from mitochondria in cells affected by KF treatment. Representative examples of SKBR3 cells stained for Hoechst (left panels) and cytochrome C (Cyt C) (right panels) incubated without (UT) or with KF (1 μM) for 2 h. The arrow in the bottom panels indicates a cell with clumped DNA but no cytochrome C release. *D,* Effect of KF on caspase-3 and PARP-cleavage. SKBR3 cells were incubated for the indicated period of time with KF and effects on caspase-3 and PARP was analysed by Western blotting. As a control, cells were treated with cisplatin (10 μM) for 24 h (lane 6). The membranes were stripped and reprobed with anti-β-actin to verify equal protein loading. *E,* No effect of caspase or cathepsin B or D inhibition on KF-induced sub-G1 population. SKBR3 cells were pretreated with the pan-caspase inhibitor zVAD-fmk (50 μM), the cathepsin B inhibitors zFA-fmk (ZFA) or CA-074 (CA), or the cathepsin D inhibitor pepstatin A (pepA) for 1 h before addition of KF (1 μM). The sub-G1 population was measured after 24 h as in *A*. Values represent the mean and SD of three independent experiments.

Annexin-V staining remained negative after treatment (Figure 1B), indicating that KF does not induce the externalization of phosphatidyl-serine (PS), which is characteristic of apoptotic cell death. KF-treatment resulted in DNA clumping, but cytochrome C remained within mitochondria even in cells clearly affected by KF-treatment (Figure 1C, indicated by the arrow). The sensitivity to KF of A549 cells overexpressing the anti-apoptotic protein Bcl-2 was similar to that of non-transfected cells (data not shown), further indicating that apoptosis was not involved in the action of KF. Neither caspase-3 nor PARP were cleaved after up to 24 h exposure to KF (Figure 1D; upper panel), and co-treatment with the broad caspase inhibitor zVAD-fmk did not prevent the hypodiploid cell population induced by KF (Figure 1E). PARP was degraded after 16 h exposure to KF, but the 89 kD caspase-dependent cleavage product that appeared in control cells treated with cisplatin, was not observed (Figure 1D; middle panel). This suggests that other proteases were involved in KF-induced degradation of PARP.

Previous reports that KF induces the loss of lysosomal integrity [21,22] and that PARP can be degraded by lysosomal cathepsins during necrosis [23] raised the possibility that the observed KF-induced PARP degradation could be mediated by these proteases, and that cathepsins might be involved in the action of KF. To investigate this possiblity, cells were co-treated with zFA-fmk or CA-074-ME, which are specific inhibitors of cathepsin B, or with the cathepsin D inhibitor pepstatin A. As shown in Figure 1E, inhibition of cathepsin B or D failed to protect from the KF-induced sub-G1 population, demonstrating that the activity of cathepsin B or D is not required for KF-mediated cytotoxicity. Taken together, these data demonstrate that KF does not activate caspase-dependent apoptosis, but causes cell death that resembles necrosis.

ErbB3 expression levels correlate with KF sensitivity

Using normal human skin fibroblasts, we confirmed previous reports [24,25] that non-tumoral cells have 5- to 40-times higher IC_{50} values for KF compared to sensitive tumor cells (data not shown). Malignant cells commonly possess over-activated signal transduction cascades that provide potential selective targets of antitumor drugs [26]. Since KF inhibits the EGFR and ErbB2 receptors and downregulates TGFα gene expression [4,27], and high expression of the ErbB

family of receptor tyrosine kinases is prevalent in tumor cells [28], we investigated if protein expression levels of ErbB receptors (ErbB1-4) correlate with the sensitivity to KF in a panel of tumor cell lines with variable expression of each receptor. The IC_{50} concentrations for KF, as measured in MTT assays, ranged from 0.1 μM to 7 μM within the panel of cell lines (Figure 2A). The sensitivity of the cell lines to KF did not correlate with their EGFR (ErbB1), ErbB2, or ErbB4 protein expression levels (Figure 2B). However, ErbB3 levels showed a significant inverse correlation (r= -0.61, P= 0.0428) with IC_{50} concentrations of KF (Figure 2B).

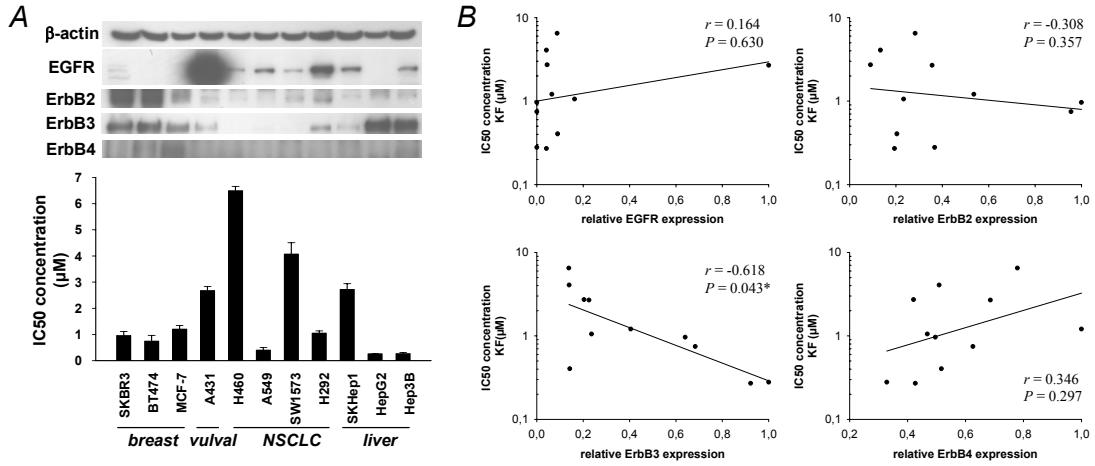


Figure 2. ErbB3 protein expression levels correlate with KF sensitivity. Correlation of KF sensitivity with expression of the ErbB family of kinases. *A,* Basal ErbB expression levels were evaluated by western blotting. IC_{50} values of KF represent the mean and SD of at least three independent MTT assays (see materials and methods). *B,* The ErbB protein expression levels shown in fig *A* were quantified using Quantity One (Biorad) software, normalized with β-actin expression levels, and plotted against the mean IC_{50} concentrations of KF. Pearson's correlation coefficients (*r*) and *P*-values were calculated using SPSS software.

KF induces downregulation of ErbB3 protein

Western blot analysis was carried out to investigate potential differences in the expression levels of ErbB receptors in the KF-sensitive HT29 colon carcinoma cell line compared to a KF-resistant sub-line (HT29/KF) generated by long term exposure to increasing concentrations of the drug, and was capable of growing in the presence of 1.3 μM KF (see Materials and Methods). The expression of all four ErbB receptors was downregulated in the KF-resistant sub-line compared to the parental cell line (Figure 3A). In contrast to the general down-regulation of ErbB family members observed after long-term exposure to KF, a 4 h treatment

with KF resulted in the selective downregulation of ErbB3 in sensitive, high ErbB3-expressing SKBR3 cells (Figure 3B).

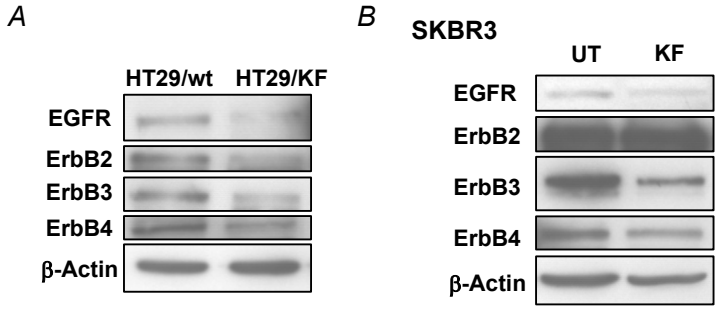


Figure 3. KF induces downregulation of ErbB3 protein. Western blot analysis of protein expression levels of ErbB receptors in *A,* HT29 wild-type (HT29/wt) and KF-resistant HT29 (HT29/KF) cells that were exposed to KF for a long period of time, and in *B,* SKBR3 cells treated with KF (1 μM) for a short period of time (4 h). The membranes were stripped and reprobed with anti-β-actin to verify equal protein loading.

Ectopic ErbB3 expression increases the sensitivity of H460 cells to KF

To investigate if ectopic ErbB3 expression affects the sensitivity of low ErbB3 expressing cells to KF treatment, H460 cells were transiently co-transfected with cDNA, encoding ErbB3 [29] and YFP as a marker of transfection. In comparison to control cells co-transfected with an empty vector and YFP, an increased percentage of YFP-positive, detached cells was noted in ErbB3 co-transfected cells upon KF treatment (data not shown). Next, H460 cells were stably transfected with an ErbB3-encoding cDNA. These cells showed increases of bands of about 125 and 30 kD, detected with an ErbB3-specific antibody, while no induction of full length ErbB3 was observed (Figure 4A, the different isoforms are indicated by arrows). Treatment of the H460-pBABE-ErbB3 cells with 2 μM KF resulted in decreased expression of the ErbB3-related 125 and 30 kD proteins (Figure 4A), similar to previously demonstrated KF-mediated depletion of full length ErbB3. In line with the results in transient transfected cells, a higher fraction of the H460-pBABE-ErbB3 cell line exhibited morphological signs of KF-induced cell death compared to control cells transfected with an empty vector (Figure 4B). Moreover, H460-pBABE-ErbB3 cells showed a modest but significant (p=0.026) reduction of the IC_{50} concentration of KF compared to empty vector-transfected cells (Figure 4C). Altogether, the results suggest that ectopic ErbB3 expression sensitizes these cells for KF treatment.

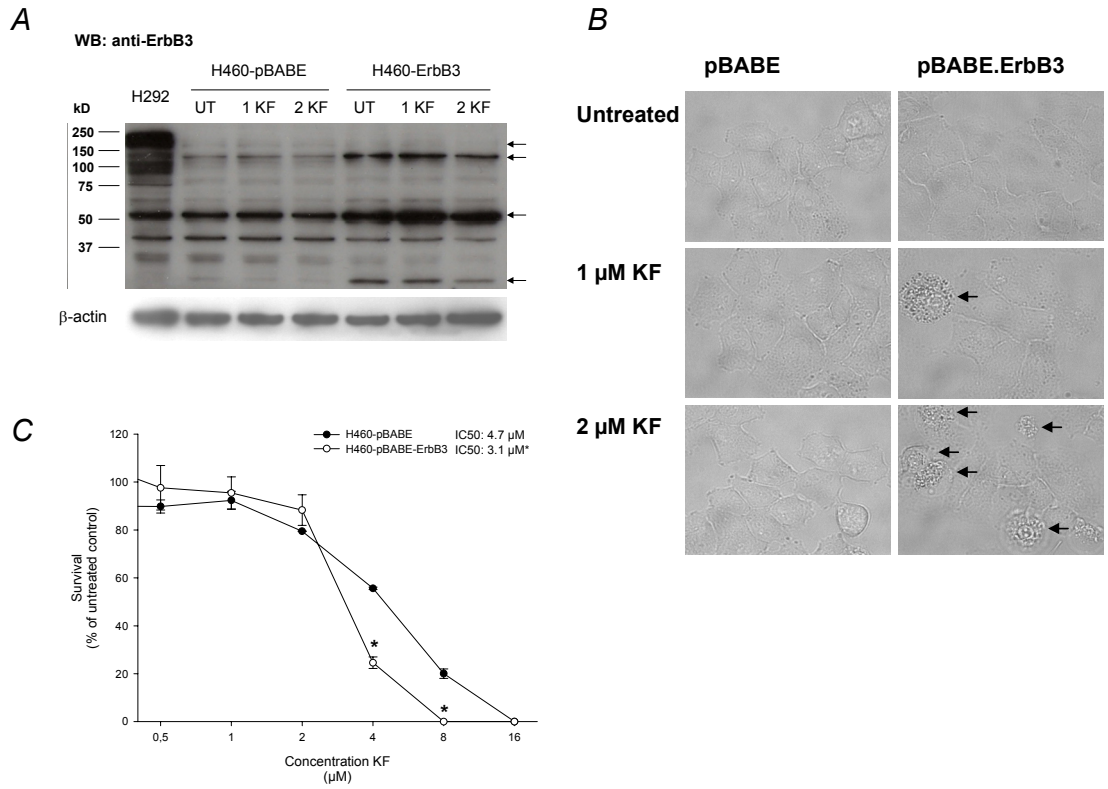


Figure 4. Ectopic ErbB3 expression increases the sensitivity of H460 cells to KF. *A,* ErbB3 protein expression was analysed by Western blotting on total cell lysates of H460 cells that were stably transfected with the empty pBABE vector or the pBABE-ErbB3 vector. Effect of KF treatment on ErbB3 expression was analyzed on cells treated with 1 or 2 μM KF for 4 h. A total cell lysate of H292 cells was included as a positive control. The arrows indicate the full length ErbB3 of 185 kD and truncated ErbB3 isoforms of about 125, 60 and 30 kD. Note the decrease of these proteins after KF treatment. *B,* Phase contrast pictures of H460 cells stably expressing the empty pBABE vector or the pBABE-ErbB3 vector, exposed to 0, 1, or 2 μM of KF for 4 h. The arrows indicate cells that were visually affected by KF treatment. *C,* H460.ErbB3 cells are more sensitive for KF treatment than H460.pBABE cells. Growth inhibition induced by various concentrations of KF was determined in MTT assays at 72 h. The percentage of survival was expressed as the percentage of the untreated controls. Mean, SD, and IC_{50} concentrations are shown from two independent experiments (*, $P \leq 0.05$, empty vector *vs* ErbB3, Student's *t* test).

KF depletes ErbB3 and inhibits Akt in KF-sensitive cells

When a panel of other tumor cell lines was analyzed, we observed that KF induced ErbB3-downregulation specifically in sensitive cell lines (Figure 5A). All ErbB receptors couple to two major signaling cascades, the MAPK pathway involving Ras/Erk, and the PI3K/Akt cascade [30], but ErbB3 is the most efficient activator of PI3K [31]. Both kinase pathways are involved in the regulation of cell proliferation and survival, and are often overactivated in tumor cells [32]. Phosphorylated or total levels of Erk were not affected by exposure of the sensitive SKBR3 cells to KF up to 24 h (Figure 5B). In contrast, phosphorylated but not total Akt levels decreased within only 30 min after addition of KF to

SKBR3 cells with the maximal effect reached after 2 h, which was sustained up to at least 24 h (Figure 5B). When the complete panel of cell lines was analyzed, we found a decrease of Akt phosphorylation only in those cell lines in which KF provoked downregulation of ErbB3 (Figure 5A), suggesting that KF-mediated Akt inhibition was due to ErbB3 depletion. In contrast, like ErbB3 expression, Akt phosphorylation remained largely unaffected in the KF-resistant cell lines, including the acquired resistant subline HT29/KF (Figure 5A). Interestingly, the basal levels of phosphorylated Akt were increased in the HT29/KF cell line compared to its parental cell line (Figure 5A).

In a titration experiment in SKBR3 cells, the decrease of phosphorylated Akt started at concentrations of 0.25 μM, having the largest effect at 1 μM (Figure 5C). Consistent with inhibition of Akt activity, the phosphorylation of the Akt substrates MDM2 as well as GSK-3β decreased in cells exposed to KF (Figure 5C). As an additional marker for Akt activity, the nuclear/cytoplasmic localization of $p27^{kip1}$ was evaluated by immunofluorescence. Nuclear $p27^{kip1}$ inhibits cyclin/CDK complexes resulting in an arrest of cells in the G1 phase of the cell cycle [33,34]. Phosphorylation of $p27^{kip1}$ by Akt results in the confinement of $p27^{kip1}$ in the cytoplasm, thus stimulating cell cycle progression [35-37]. Treatment with KF induced redistribution of $p27^{kip1}$ from the cytosol to the nucleus to a similar extent as the selective PI3K inhibitor LY294002, included as a control, further demonstrating that Akt activity was inhibited (Figure 5D).

Cells treated with KF exhibited extensive cytoplasmic swelling cells and rapidly detached from the bottom of the tissue culture tray. After treatment with KF for 1 h, detached cells were separately harvested from attached cells. Downregulation of ErbB3 and inhibition of Akt was only observed in detached cells (Figure 5E), indicating that these events coincide with KF-induced cytotoxicity.

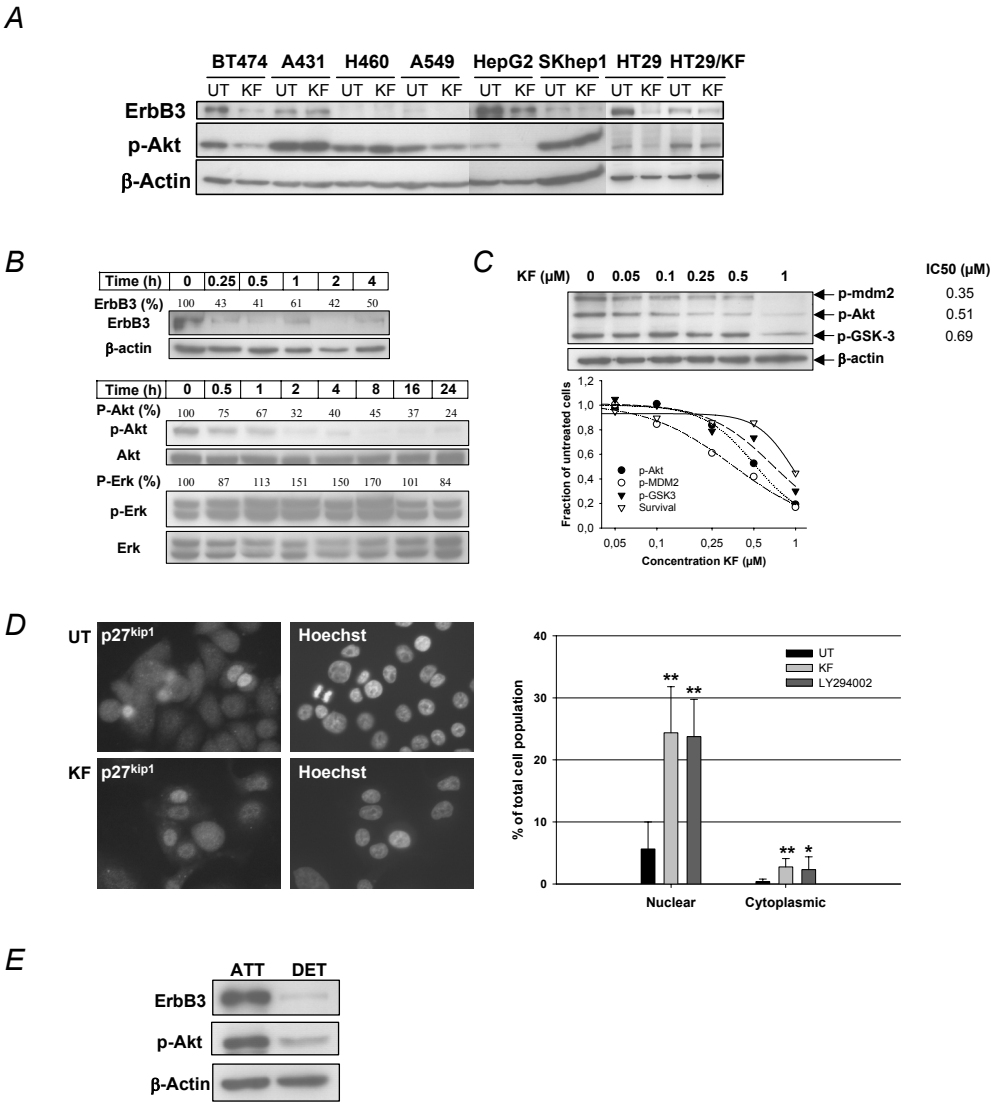


Figure 5. KF depletes ErbB3 and inhibits Akt in sensitive cells. *A,* The indicated cell lines were left untreated (UT) or exposed to KF (1 μM) for 4 h, and ErbB3 and p (phosphorylated)-Akt expression levels were analyzed by Western blotting. The membrane was stripped and reprobed with anti-β-actin to verify equal protein loading. *B,* SKBR3 cells were exposed to KF (1 μM) for different times and ErbB3 and total and phosphorylated (p) Erk and Akt were analysed by Western blotting. Protein expression levels of ErbB3, p-Akt, and p-Erk were quantified, normalized with β-actin, and calculated as percentage of untreated cells. *C,* SKBR3 cells were exposed to different concentrations of KF as indicated, and phosphorylated (p) Akt, mdm2, and GSK-3β were analysed by Western blotting. The concentrations of KF resulting in 50% inhibition of protein expression (IC50) were calculated after quantification of the Western blots. The lower panel shows the quantified and normalized protein expression levels of p-Akt, p-MDM2 and p-GSK3β plotted against the KF concentration. The graph also shows the survival of SKBR3 cells after 72 h treatment plotted against the KF concentration as measured in MTT assays (see materials and methods). *D,* Representative examples of SKBR3 cells incubated without (UT) or with KF (1 μM) for 4 h and stained for $p27^{kip1}$ and Hoechst. The proportion of cells showing predominantly nuclear localization of $p27^{kip1}$, or predominant cytoplasmic $p27^{kip1}$ localization is shown in the bottom panel, including of cells that were treated with the PI3K inhibitor LY294002, used as a control. The proportion of cells that had both nuclear and cytoplasmic $p27^{kip1}$ localization is not shown. Values represent the mean and SD by counting at least 200 cells/treatment in two independent experiments (*, $P \leq 0.05$; **, $P \leq 0.01$, untreated *vs* treated, Student's *t* test). *E,* SKBR3 cells were exposed for 1 h with KF (1 μM) and the attached and detached cell populations were separately harvested and analyzed for ErbB3 and phosphorylated Akt protein levels.

Constitutive activation of Akt protects SKBR3 cells from KF-induced cytotoxicity

KF-mediated Akt dephosphorylation requires the activity of protein phosphatases PP2A and/or PP1, as pre-treatment with the phosphatase inhibitor okadaic acid (OA) prevented KF-induced Akt dephosphorylation (data not shown). However, OA failed to protect against KF-induced cytotoxicity (data not shown), probably due to additional toxic effects of PP1 and PP2A inhibition. Hence, as another approach to prevent KF-induced inactivation of Akt, cells were transfected with a plasmid encoding for gag-PKB, a constitutively active mutant of Akt [38]. A smaller number of cells that were transiently co-transfected with gag-PKB and YFP exhibited morphological signs of KF-induced cell death compared to cells transfected with YFP alone (Figure 6A). As shown in Figure 6B, co-transfection with gag-PKB significantly reduced the percentage of YFP-positive detached cells upon KF treatment, indicating that constitutively active Akt protects against KF-cytotoxicity.

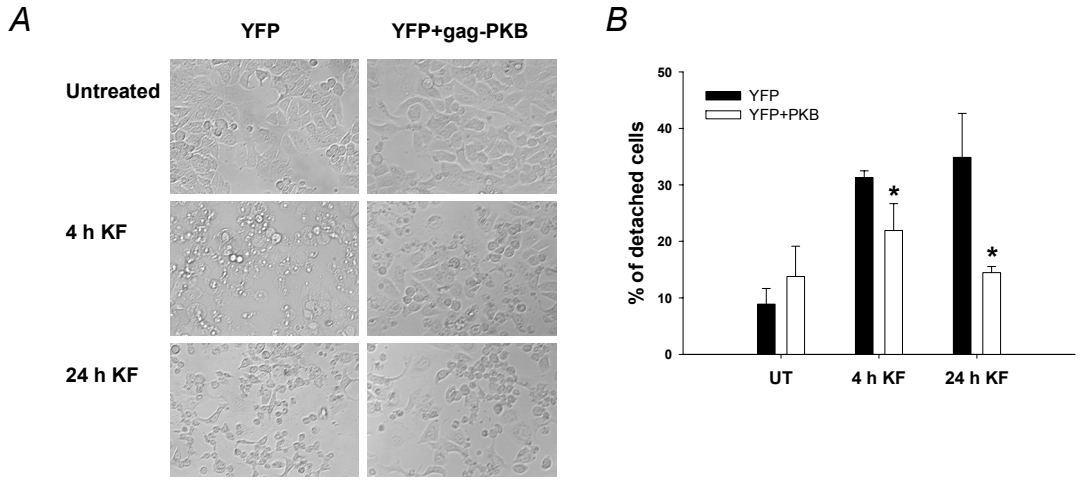


Figure 6. Constitutive activation of Akt protects SKBR3 cells from KF-induced cytotoxicity. *A,* Phase contrast pictures of cells transfected with YFP alone (YFP) or YFP and gag-PKB (YFP+gag-PKB), which were left untreated or exposed to KF (1 μM) for 4 or 24 h. *B*, Fraction of YFP-positive cells that had detached from the bottom of the tissue culture tray after exposure to KF (1 μM) for 4 or 24 h. Mean and SD are shown from two independent assays by counting at least 300 cells/treatment (*, $P \leq 0.05$, YFP *vs* YFP+gag-PKB, Student's *t* test).

Discussion

In the study presented here, we have investigated the molecular mechanism of action of the novel marine ant-cancer compound KF, which is currently being investigated in phase II clinical trials. In line with other studies [39,40], we observed rapid and potent cytotoxic activity against ErbB2-overexpressing breast cancer cell lines, which was associated with the induction of a hypodiploid cell population, dramatic cytoplasmic swelling, and permeabilization of the plasma and lysosomal membranes. The cell death induced by KF cannot be classified as apoptosis, as several apoptotic markers were negative after KF exposure, and molecular and chemical inhibitors of caspase-dependent cell death failed to protect against KF. In addition to caspase-mediated apoptosis, cells can activate alternative types of programmed cell death mediated by other proteases, such as calpains and cathepsins [41]. However, specific inhibitors of the lysosomal proteases cathepsin B or D (Figure 1E) or of the protease calpain (data not shown) also failed to protect against KF-induced cytotoxicity. In line with a previous study in prostate and breast cancer cells [42], our data indicate that KF induces a necrosis-like cell death process.

We provide several lines of evidence that point to ErbB3 as a major determinant of KF action. First, the inverse correlation between ErbB3 expression and KF IC_{50} values within the panel of human tumor cell lines suggests ErbB3 as a marker for KF-sensitivity. Second, exposure to KF results in downregulation of ErbB3 protein expression, which was observed in cells exposed for a short time (4 h) to KF as well as in KF-resistant cells that were selected over a longer period of time in KF-containing medium. Third, KF treatment induced downregulation of ErbB3 primarily in the detached, dying cell fraction, but not in the attached cell fraction. Finally, H460 cells ectopically expressing ErbB3 were more sensitive for KF treatment than cells transfected with an empty vector. Together, this indicates that downregulation of ErbB3 in cells that depend on ErbB3 for their survival contributes to the cytotoxicity of KF.

Interestingly, H460 cells transfected with full length ErbB3 cDNA expressed a smaller, 125 kD protein. Others have demonstrated that ErbB3 exists in several isoforms of different sizes, including the truncated extracellular domain of about 30 kD in size, which is located as a 58 kD disulphide-linked dimer in vesicles in

the cytoplasm [43,44]. However, the 125 kD protein that we also observed has not been described. Under-glycosylation or proteolytic cleavage of the full-length protein may potentially account for the reduced ErbB3 protein size in H460 cells. In turn, low expression levels of full-length, functional ErbB3 is one of the factors that may account for the limited effect of ErbB3 transfection. Several other factors, however, might contribute to the relatively small differences between the IC_{50} values of ErbB3- and empty vector-transfected H460 cells. On one hand, since ErbB3 lacks intrinsic kinase activity, functional ErbB3 also requires the expression of other ErbB receptors, which is poor in H460 cells (see Figure 2). On the other hand, the use of pooled transfectants may have masked larger cytotoxic effects induced by KF, as a subpopulation of the cells may express only small amounts of ErbB3.

KF-mediated depletion of ErbB3 was accompanied by a rapid decrease in Akt phosphorylation and inhibition of downstream signaling, an effect that was only observed in sensitive, ErbB3-expressing cells. This finding, together with our observation that blockage ofthe PI3K/Akt pathway with the PI3K inhibitor LY294002 did not affect ErbB3 expression (data not shown), suggests that inhibition of Akt signaling reflects KF-mediated ErbB3 depletion. This is also consistent with the notion that functional ErbB3 is required for Akt activity in cells that depend on ErbB2 and ErbB3 for their growth and survival, such as SKBR3 cells [45].

Our data indicate that KF-mediated inhibition of the ErbB3-Akt pathway contributes to KF cytotoxicity, as cells transfected with a constitutively active mutant of Akt were largely protected against KF-cytotoxicity. Moreover, the KF-resistant cell line HT29/KF showed increased basal Akt phosphorylation compared to its parental, KF-sensitive cell line HT29, which was not decreased after KF exposure. However, no KF-mediated inhibition of Akt phosphorylation was observed in ErbB3-transfected H460 cells (data not shown). Akt activity is partially uncoupled from growth factor receptor activity in H460 cells (Janmaat et al, 2003), providing a possible explanation for the observation that ErbB3 depletion does not affect Akt activity in these cells. Akt is a major downstream target of receptor tyrosine kinases that signal via PI3K [46], and possesses pro-survival and anti-apoptotic activities [47]. Although the role of Akt in apoptosis suppression is well established [48], little is known about the involvement of Akt in

necrosis-like cell death. Akt inactivation has been observed in multiple types of caspase-independent cell death induced by agents such as N-methyl-D-aspartate, nitric oxide, hydrogen peroxidase, and ansamycin antibiotics [49,50]. Similar to our results with KF, the introduction of a constitutively active mutant of Akt suppressed N-methyl-D-aspartate toxicity [51]. However, the exact underlying mechanism remains to be investigated.

The demonstration that ErbB3 expression levels correlated with KF sensitivity may explain the reported preferential effect of KF on tumor versus normal cells [52,53], as ErbB3 is commonly overexpressed in tumors [54]. However, the NSCLC cell line A549, with low ErbB3 levels, was highly sensitive for KF, suggesting that ErbB3 expression levels alone are not predictive of KF sensitivity in all cases. Although A549 cells show low ErbB3 expression, their survival is dependent on ErbB3 expression, as selective ErbB3 depletion using RNA interference has been shown to induce cell death in this cell line [55].

In contrast to an earlier observation of KF-mediated inhibition of EGFR and ErbB2 [4,56], we did not detect any significant, direct effect of KF treatment on EGFR or ErbB2 expression (Figure 3) or phosphorylation in A431 or SKBR3 cells, respectively (data not shown). However, the observation that besides ErbB3 other ErbB receptors were also downregulated to some extent during the selection process of the KF-resistant HT29 subline, might reflect an indirect effect of KF on the expression of other ErbB receptors, and suggests a potential role of multiple members of the ErbB family in KF-sensitivity.

The mechanism by which ErbB3 is downregulated by KF remains to be clarified. KF-induced downregulation of ErbB3 is not due to inhibited synthesis of the receptor, as treatment of SKBR3 cells with the protein synthesis inhibitor cyclohexamide (50 μM) for 4h did not affect ErbB3 expression levels, while expression of $p27^{kip1}$ was downregulated in these cells (data not shown). These data thus indicate that ErbB3 downregulation is due to degradation rather than inhibition of protein synthesis. ErbB3 protein can be ubiquitinated by the ubiquitin ligase Nrpd1 and subsequently degraded by proteasomes [57,58]. However, KF-induced ErbB3-degradation was not proteasome-mediated, as co-treatment of cells with the proteasome inhibitors MG-132 or PS-341 failed to protect from KF-induced ErbB3 depletion (data not shown). Alternatively, KF might mediate the internalization of ErbB3 and subsequently target it for

degradation, similar to EGF-mediated internalization of the EGFR [59]. However, it remains to be elucidated if and how KF binds ErbB3.

The data presented here may have important clinical relevance. We demonstrate that KF is active *in vitro* against cells derived from various tumor types, including breast, colon, NSCLC, and hepatic carcinomas at clinically relevant concentrations. Moreover, our finding that ErbB3 expression levels correlate with sensitivity of cell lines to KF suggests ErbB3 as a marker for KF sensitivity. Furthermore, downregulation of ErbB3 expression or inhibition of Akt or downstream events, such as the cellular localization of $p27^{kip1}$, could serve as surrogate markers for KF activity. Although these preclinical findings require confirmation in the clinic, our data further suggest that mutations leading to constitutive activation of the PI3K/Akt pathway, such as depletion of *PTEN* [60] or *PIK3CA* mutations [61] that have been identified in several tumor types, may contribute to mechanisms of resistance to KF.

References

1. Hamann, MT *et al.* Kahalalide F: a bioactive depsipeptide from the sacoglossan mollusk *Elysia rufescens* and the green alga *Bryopsis* sp. *J Am Chem Soc* **115**, 5825-5826 (1993).

2. Hamann, MT *et al.* Kahalalides: Bioactive Peptides from a Marine Mollusk Elysia rufescens and Its Algal Diet Bryopsis sp.(1). *J Org Chem* **61**, 6594-6600 (1996).

3. Faircloth, GT *et al.* Preclinical development of Kahalalide F, a new marine compound selected for clinical studies. *Proc Am Assoc Cancer Res* **41**, 600 (2000).

4. Faircloth, GT *et al.* Selective antitumor activity of Kahalalide F, a marine-derived cyclic depsipeptide. *Proc Am Assoc Cancer Res* **42**, 213 (2001).

5. Jimeno, JM *et al.* Progress in the acquisition of new marine-derived anticancer compounds: development of ecteinascidin-743 (ET-743). *Drugs Future* **21**, 1155-1165 (1996).

6. Medina, LA *et al.* Investigation of the effects of Kahalalide F (PM92102) against tumor specimens taken directly from patients. *Proc Am Assoc Cancer Res* **42**, 213 (2001).

7. Shao, L *et al. In vitro* anti-proliferative effect on sarcoma cells of ET-743 and other marine chemotherapeutics. *Proc Am Assoc Cancer Res* **42**, 203 (2001).

8. Suarez, Y *et al.* Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. *Mol Cancer Ther* **2**, 863-872 (2003).

9. Gomez, SG *et al.* In vitro toxicity of three new antitumoral drugs (trabectedin, aplidin, and kahalalide F) on hematopoietic progenitors and stem cells. *Exp Hematol* **31**, 1104-1111 (2003).

10. Ciruelos, C *et al.* A phase I clinical and pharmokinetic (PK) study with Kahalalide F (KF) in patients (pts) with advanced solid tumors (AST) with a continuous weekly (W) 1-hour iv infusion schedule. *Eur J Cancer* **38 (Suppl. 7)**, S33 (2002).

11. Jimeno, J *et al.* Progress in the clinical development of new marine-derived anticancer compounds. *Anticancer Drugs* **15**, 321-329 (2004).

12. Garcia-Rocha, M *et al.* The antitumoral compound Kahalalide F acts on cell lysosomes. *Cancer Lett* **99**, 43-50 (1996).

13. Suarez, Y *et al.* Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. *Mol Cancer Ther* **2**, 863-872 (2003).

14. Suarez, Y *et al.* Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. *Mol Cancer Ther* **2**, 863-872 (2003).

15. Wosikowski, K *et al.* Identification of epidermal growth factor receptor and c-erbB2 pathway inhibitors by correlation with gene expression patterns. *J Natl Cancer Inst* **89**, 1505-1515 (1997).

16. Suarez, Y *et al.* Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. *Mol Cancer Ther* **2**, 863-872 (2003).

17. Janmaat, ML *et al.* Response to epidermal growth factor receptor inhibitors in non-small cell lung cancer cells: limited antiproliferative effects and absence of apoptosis associated with persistent activity of extracellular signal-regulated kinase or Akt kinase pathways. *Clin Cancer Res* **9**, 2316-2326 (2003).

18. Nicoletti, I *et al.* A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* **139**, 271-279 (1991).

19. Holbro, T *et al.* The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci U S A* **100**, 8933-8938 (2003).

20. Burgering, BM *et al.* Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599-602 (1995).

21. Garcia-Rocha, M *et al.* The antitumoral compound Kahalalide F acts on cell lysosomes. *Cancer Lett* **99**, 43-50 (1996).

22. Suarez, Y *et al.* Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. *Mol Cancer Ther* **2**, 863-872 (2003).

23. Gobeil, S *et al.* Characterization of the necrotic cleavage of poly(ADP-ribose) polymerase (PARP-1): implication of lysosomal proteases. *Cell Death Differ* **8**, 588-594 (2001).

24. Gomez, SG *et al.* In vitro toxicity of three new antitumoral drugs (trabectedin, aplidin, and kahalalide F) on hematopoietic progenitors and stem cells. *Exp Hematol* **31**, 1104-1111 (2003).

25. Suarez, Y *et al.* Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. *Mol Cancer Ther* **2**, 863-872 (2003).

26. Blume-Jensen, P *et al.* Oncogenic kinase signalling. *Nature* **411**, 355-365 (2001).

27. Wosikowski, K *et al.* Identification of epidermal growth factor receptor and c-erbB2 pathway inhibitors by correlation with gene expression patterns. *J Natl Cancer Inst* **89**, 1505-1515 (1997).

28. Salomon, DS *et al.* Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* **19**, 183-232 (1995).

29. Holbro, T *et al.* The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci U S A* **100**, 8933-8938 (2003).

30. Yarden, Y *et al.* Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2**, 127-137 (2001).

31. Prigent, SA *et al.* Identification of c-erbB-3 binding sites for phosphatidylinositol 3'-kinase and SHC using an EGF receptor/c-erbB-3 chimera. *EMBO J* **13**, 2831-2841 (1994).

32. Blume-Jensen, P *et al.* Oncogenic kinase signalling. *Nature* **411**, 355-365 (2001).

33. Toyoshima, H *et al.* p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* **78**, 67-74 (1994).

34. Polyak, K *et al.* Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78**, 59-66 (1994).

35. Viglietto, G *et al.* Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. *Nat Med* **8**, 1136-1144 (2002).

36. Liang, J *et al.* PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat Med* **8**, 1153-1160 (2002).

37. Shin, I *et al.* PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. *Nat Med* **8**, 1145-1152 (2002).

38. Burgering, BM *et al.* Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599-602 (1995).

39. Garcia-Rocha, M *et al.* The antitumoral compound Kahalalide F acts on cell lysosomes. *Cancer Lett* **99**, 43-50 (1996).

40. Suarez, Y *et al.* Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. *Mol Cancer Ther* **2**, 863-872 (2003).

41. Leist, M *et al.* Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* **2**, 589-598 (2001).

42. Suarez, Y *et al.* Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. *Mol Cancer Ther* **2**, 863-872 (2003).

43. Srinivasan, R *et al.* Intracellular expression of the truncated extracellular domain of c-erbB-3/HER3. *Cell Signal* **13**, 321-330 (2001).

44. Lee, H *et al.* Isolation and characterization of four alternate c-erbB3 transcripts expressed in ovarian carcinoma-derived cell lines and normal human tissues. *Oncogene* **16**, 3243-3252 (1998).

45. Holbro, T *et al.* The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci U S A* **100**, 8933-8938 (2003).

46. Burgering, BM *et al.* Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599-602 (1995).

47. Franke, TF *et al.* PI3K/Akt and apoptosis: size matters. *Oncogene* **22**, 8983-8998 (2003).

48. Franke, TF *et al.* PI3K/Akt and apoptosis: size matters. *Oncogene* **22**, 8983-8998 (2003).

49. Luo, HR *et al.* Akt as a mediator of cell death. *Proc Natl Acad Sci U S A* **100**, 11712-11717 (2003).

50. Basso, AD *et al.* Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2. *Oncogene* **21**, 1159-1166 (2002).

51. Luo, HR *et al.* Akt as a mediator of cell death. *Proc Natl Acad Sci U S A* **100**, 11712-11717 (2003).

52. Gomez, SG *et al.* In vitro toxicity of three new antitumoral drugs (trabectedin, aplidin, and kahalalide F) on hematopoietic progenitors and stem cells. *Exp Hematol* **31**, 1104-1111 (2003).

53. Suarez, Y *et al.* Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. *Mol Cancer Ther* **2**, 863-872 (2003).

54. Salomon, DS *et al.* Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* **19**, 183-232 (1995).

55. Sithanandam, G *et al.* Inactivation of ErbB3 by siRNA blocks growth and promotes apoptosis in human lung adenocarcinoma cell line A549. *Proc Am Assoc Cancer Res* **45**, 2426 (2004).

56. Wosikowski, K *et al.* Identification of epidermal growth factor receptor and c-erbB2 pathway inhibitors by correlation with gene expression patterns. *J Natl Cancer Inst* **89**, 1505-1515 (1997).

57. Diamonti, AJ *et al.* An RBCC protein implicated in maintenance of steady-state neuregulin receptor levels. *Proc Natl Acad Sci U S A* **99**, 2866-2871 (2002).

58. Qiu, XB *et al.* Nrdp1/FLRF is a ubiquitin ligase promoting ubiquitination and degradation of the epidermal growth factor receptor family member, ErbB3. *Proc Natl Acad Sci U S A* **99**, 14843-14848 (2002).

59. French, AR *et al.* Intracellular trafficking of epidermal growth factor family ligands is directly influenced by the pH sensitivity of the receptor/ligand interaction. *J Biol Chem* **270**, 4334-4340 (1995).

60. Stambolic, V *et al.* Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95**, 29-39 (1998).

61. Samuels, Y *et al.* High frequency of mutations of the PIK3CA gene in human cancers. *Science* **304**, 554 (2004).

Chapter 6

Summarizing discussion

The major aim of the investigation described in this thesis was to further understand the molecular mechanisms of ErbB-targeting anti-tumour agents, and to identify biological markers that predict the outcome of cancer patients treated with such agents.

To address these issues, we started to investigate the molecular mechanism of action of the EGFR-TKI gefitinib and the EGFR antagonistic antibody C225. Despite the promising activity seen in several NSCLC patients in early clinical trials with gefitinib [1-3], we observed only small anti-proliferative effects of anti-EGFR agents in NSCLC cell lines *in vitro*. On the contrary, the growth of the EGFR-overexpressing cell line A431 was effectively inhibited due to an arrest of cells in the G1-phase of the cell cycle (*Chapter 2*). Correspondingly, the survival of NSCLC cells remained unaffected upon treatment with EGFR inhibitors, whereas apoptotic cell death was induced in A431 cells. As may have been expected (Figure 1), we demonstrated that apoptosis induced by EGFR inhibitors involved a mitochondrial step (*Chapter 2*). However, apoptotic cell death induced by EGFR inhibitors has only been observed in some tumour cells [4-10], indicating that the induction of apoptosis is not a general mechanism of action of this type of agents.

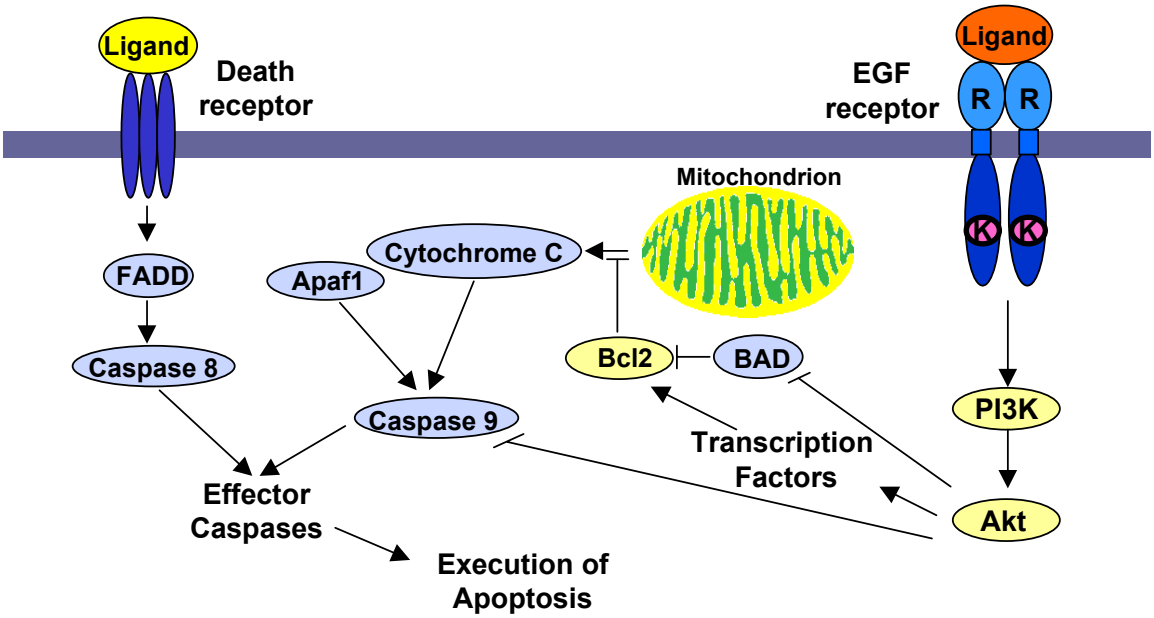
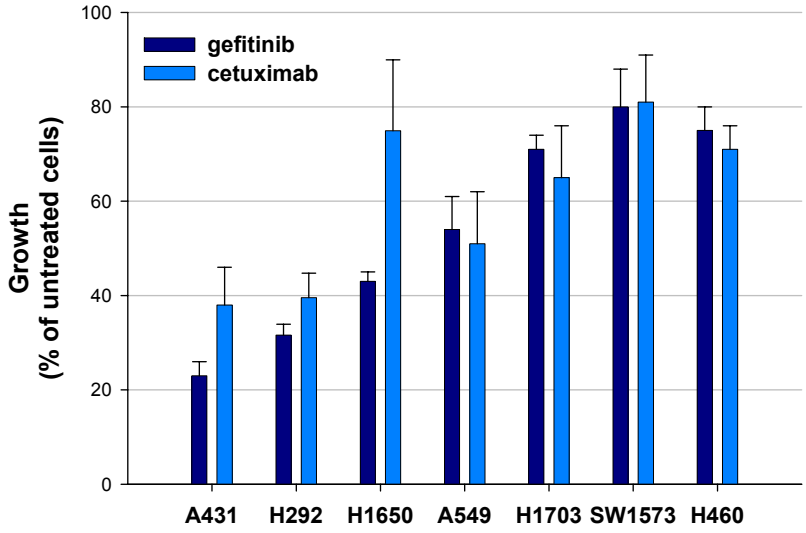


Figure 1. Interference of the EGFR pathway on apoptosis. Apoptosis can be induced via two distinct pathways. Activation of death receptors by their ligands results in apoptotic cell death via subsequent activation of FADD, Caspase 8, and downstream effector caspases, such as Caspase 3. On the other hand, DNA damage induced by for instance chemotherapy may result in the release of Cytochrome C from mitochondria, which together with the co-factor Apaf1 activates Caspase 9. Activated Caspase 9 induces downstream activation of effector caspases, resulting in the execution of apoptosis. Activation of the EGFR pathway has an inhibitory effect on Caspase 9 mediated apoptosis by activating the PI3K-Akt pathway, which results in the retention of Cytochrome C in mitochondria mediated by anti-apoptotic Bcl2 family members. Alternatively, Akt is known to directly inhibit Caspase 9 activity.

Next, we sought to resolve which factors determine anti-EGFR sensitivity and resistance. We hypothesized that tumour cells that strongly depend on the EGFR pathway for their growth and survival would be most sensitive to EGFR inhibitors. Initially, we and others found that EGFR expression levels do not directly correlate with sensitivity to EGFR inhibitors [5,11,12]. However, the discovery of *EGFR* kinase domain mutations provided an entirely new perspective in the research field. The finding that the presence of these mutations strongly correlates with gefitinib-sensitivity [13-15] also lead to the novel appreciation of the importance of EGFR expression levels, particularly due to gene amplification, as a marker of anti-EGFR activity in *EGFR* wild-type cell lines and tumours [16-18] (*Chapter 3 and 4*). For instance, we showed that high EGFR protein expression in esophageal tumours, in which no mutations in the EGFR tyrosine kinase domain were found or reported, predicts for increased sensitivity to gefitinib treatment (*Chapter 4*). Together, these data suggest that EGFR expression levels may be related to EGFR-TKI sensitivity of wild type EGFR tumours, including wild type EGFR NSCLCs. However, discrepancy exists in the literature about the role of EGFR (over)expression as marker of anti-EGFR therapy or as prognostic factor in some tumour types, in large part because of the wide variety in detection methods and cut-offs used to define overexpression (reviewed in [19]). In this respect, analysis of gene amplification would be a reproducible and objective method. However, high EGFR expression does not appear to be the result of gene amplification in all cases [17] (*Chapter 4*). Thus, in order to use EGFR as marker for anti-EGFR therapy or as prognostic factor it will be important to introduce a widely accepted standard test to evaluate EGFR protein expression levels in tissues, similar to the standardized investigation of Her2/neu expression in breast cancer (DAKO HercepTest™) to identify candidates for treatment with trastuzumab [20].

The finding that *EGFR* kinase domain mutations in NSCLCs are associated with EGFR-TKI sensitivity is definitively a major breakthrough [13-15]. Indeed, many retrospective, follow-up studies have confirmed this association in NSCLCs, including a study in our group [21] (M.I. Gallegos-Ruiz *et al.*, unpublished data). However, fundamental differences appear to exist between gefitinib and erlotinib with respect to *EGFR* mutations. While 46-80% of NSCLC patients with *EGFR* mutations respond to gefitinib monotherapy [16,17], only about 15% of patients with *EGFR* mutations had a response upon erlotinib treatment [18,22]. Based on these data, the importance of *EGFR* mutations as a predictive factor for sensitivity to EGFR-TKIs in NSCLC is currently



Mutational status								
	EGFR	WT	WT	MUT (delE746-A750)	WT	WT	WT	WT
	K-ras	WT	WT	WT	MUT (G12S)	WT	MUT (G12C)	MUT (G61H)

Figure 2. Correlation between the mutational status of *EGFR* and *k-ras* and sensitivity for gefitinib or cetuximab in a panel of NSCLC cell lines and A431 vulval carinoma cells. Cell growth was measured in MTT assays after 72 h treament. Note that the cell line with an *EGFR* mutation is sensitive for treatment with gefitinib but not cetuximab. Cell lines with *k-ras* mutations are less sensitive to treatment with gefitinib or cetuximab.

evaluated in prospective studies with gefitinib and further retrospective studies with erlotinib.

In contrast to EGFR-TKIs, recent data, including from our lab, suggest that the EGFR-specific mAb cetuximab does not effectively the viability of EGFR-mutated tumour cells [23,24] (Figure 2). Apparently, extracellular antibody binding of EGFR does not affect the proliferative and pro-survival potential of the mutated EGFR.

In addition to EGFR expression, amplification, and mutations, the impact of Erk and Akt phosphorylation status has been extensively studied. We were among the first to show that EGFR-independent activity of the Erk and Akt pathways confers resistance of tumour cells to anti-EGFR agents *in vitro* (*Chapter 2*). EGFR-independent activity of these kinase pathways can be mediated by multiple factors, including PTEN depletion and *k-ras* mutations, which have been directly associated with gefitinib resistance *in vitro* and in patients [25-27] (*Chapter 3 and 4*). Positive immunohistochemical staining of NSCLC tissue for phosphorylated Akt but not phosphorylated Erk has been associated with sensitivity of patients to gefitinib by Cappuzzo *et al* [28]. However, we have not observed such correlation in the study investigating gefitinib in esophageal cancer (*Chapter 4*). These differences may reflect dissimilarities between the tumour types. On the other

hand, as with EGFR stainings, the discrepancy may be due to differences in scoring methods. In fact, Cappuzzo *et al* used a method for scoring phosphorylated Akt staining that was based on the presence of nuclear staining [28], whereas we used a method that was based on staining incidence and intensity. A complicating factor is that we were not able not score a substantial amount of esophageal tissues for phosphorylated Akt due to staining of normal tissue surrounding the tumour cells in (*Chapter 4*). In most immunohistochemical studies, including ours, only one Akt phosphorylation site (S473) has been evaluated. However, a recent report suggests that the evaluation of the two phosphorylation sites (T308 and S473) that are required for full Akt activation improves the prognostic significance of Akt activation [29].

In addition to the primary resistance related to the presence of *k-ras* mutations or EGFR-independent activity of Erk or Akt pathways, acquired resistance to erlotinib or gefitinib has been associated with a secondary point mutation in the EGFR tyrosine kinase domain [30,31]. In addition to acquired resistance, the T790M mutation has been found to be present at the time of diagnosis in several studies [32,33]. The finding that treatment with several ErbB-TKIs can overcome resistance associated with a secondary mutation [34,35] suggests that second-generation EGFR-TKIs can be of importance in the treatment of NSCLC. Strikingly, the identified second-generation EGFR-TKIs were all irreversible inhibitors of EGFR in contrast to gefitinib and erlotinib, suggesting improved efficacy of irreversible TKIs against the resistant EGFR mutant in comparison with reversible TKIs.

Altogether, the above-described data are in line with our initial hypothesis that cells that strongly depend on EGFR signalling for their growth and survival are sensitive to EGFR antagonists. Targeting kinases on which cancer cells strongly depend for their proliferation and survival, a phenomenon also referred to as kinase addiction [36], has been proven to be a successful anti-cancer strategy in several cases. In addition to successful treatment of EGFR-dependent lung tumours with EGFR tyrosine kinase inhibitors, patients with chronic myeloid leukaemia (CML) aberrantly expressing the BCR-ABL fusion protein can be successfully treated with the tyrosine kinase inhibitor imatinib (Gleevec™) [37]. Other examples of successful treatment of kinase-addicted tumours are therapy of ErbB2-overexpressing breast cancer patients with the anti-ErbB2 antibody trastuzumab (Herceptin™) [38], and treatment of patients with gastro-intestinal stromal tumours (GIST) with activating c-kit or PDGFR mutations with the multi-targeting tyrosine kinase inhibitor imatinib [39,40]. Moreover, the similarity between

imatinib and EGFR-TKIs extents to the ultimate development of acquired resistance that have been associated with secondary mutations in target genes in both cases [30,31,41,42].

Besides the molecular markers of sensitivity to EGFR-TKIs that have been described above, several clinical parameters have been put forward that correlate with EGFR-TKI sensitivity in NSCLC patients, which include female gender, Asian ethnicity, non-smoking history, and adenocarinoma histology [18,43-45]. In contrast to NSCLCs, we found that SCC histology is a predictive factor for beneficial outcome of gefitinib-treated esophageal cancer patients (*Chapter 4*). The higher incidence of EGFR mutations in lung adenocarcinomas compared to SCCs is likely to be the reason why lung cancer patients with adenocarcinoma histology respond better to gefitinib. In contrast to NSCLCs, no EGFR mutations are present in esophageal carcinomas. Esophageal SCC patients may have a better outcome than esophageal adenocarcinoma patients due to a higher rate of EGFR overexpression in SCCs (*Chapter 4*).

An issue that needs to be addressed includes the question if broader selectivity of EGFR-TKIs impacts the anti-tumor efficacy. For instance, objective and minor responses were achieved with the EGFR/ErbB2 inhibitor GW-572016 in patients pretreated with gefitinib or trastuzumab, indicating that a broader specificity may be more effective in some cases. However, a major drawback is the higher toxicity observed in patients treated with agents targeting multiple ErbB receptors, possibly due to nonspecific side effects. An alternative may be treatment with combinations of highly specific agents, such as combined treatment with gefitinib and trastuzumab, which has been successfully assessed in patients with advanced breast cancer [46]. Several reports demonstrated that combination treatment with gefitinib and cetuximab may be an effective strategy [47,48]. The recent observation that two patients responded to gefitinib after failure of several chemotherapy regimens and cetuximab [49] favors the view that monoclonal antibodies and TKIs have different mechanisms of action and might be effectively combined. On the other hand, we demonstrated in *Chapter 3* that combinations of gefitinib and specific inhibitors of the Ras/Erk and PI3K/Akt pathways induced enhanced cytotoxicity in NSCLC cells *in vitro*. These data were obtained in cell lines with EGFR-independent activity of the Ras/Erk and PI3K/Akt pathways, respectively. The results described above are examples of effective cell-specific treatment combinations, resulting from molecular characterization of specific cellular defects. When extrapolated to the treatment of patients, one may aim to achieve

patient-specific treatment with cocktails of targeted agents in the future, in contrast to the current tumor-type guided treatment with non-specific cytotoxic agents.

In addition to rationally developed selective EGFR inhibitors, we have investigated the molecular mechanism of action of the marine-derived depsi-peptide Kahalalide F. KF has specific and potent preclinical anti-tumour activity in vitro and in vivo [50-54]. KF rapidly induces disruption of membranes, particularly lysosomal membranes, followed by caspase-independent cell death [55,56] (*Chapter 5*). Initially, KF has been described to inhibit the activation of EGFR and ErbB2 and to downregulate TGFα [51,57]. In contrast to EGFR or ErbB2, we have provided several lines of evidence that point to ErbB3 as a major determinant of KF action. First, we have found an inverse correlation between ErbB3 expression and KF sensitivity within a panel of human tumour cell lines. Second, exposure to KF results in down-regulation of ErbB3 protein expression, which was observed in cells exposed for a short time (4 h) to KF and in KF-resistant cells that were selected over a longer period of time in KF-containing medium. Third, KF treatment induced down-regulation of ErbB3 primarily in cells that were detached from the bottom of the tissue culture plastic and were presumed to be dead, but not in cells that remained attached and were viable. Finally, the most resistant cell line in our panel that ectopically expressed ErbB3 became more sensitive for KF treatment than cells transfected with an empty control vector. Together, this indicates that down-regulation of ErbB3 in cells that depend on ErbB3 for their survival contributes to the cytotoxicity of KF.

KF-mediated depletion of ErbB3 was accompanied by a rapid decrease in Akt phosphorylation and inhibition of downstream signalling, an effect that was only observed in sensitive, ErbB3-expressing cells. This suggests that inhibition of Akt signalling reflects KF-mediated ErbB3 depletion, in line with other reports showing the requirement of ErbB3 for PI3K-Akt signalling [58]. Conversely, ectopic expression of a constitutively active Akt mutant in a cell line sensitive to KF largely protected against KF-induced cytotoxicity. In summary, our results identify ErbB3 and the downstream PI3K-Akt pathway as important determinants of the cytotoxic activity of KF *in vitro* (*Chapter 5*).

In line with previous data, Sewell *et al* recently showed that the pattern of cell permeability induced by KF is similar to maitotoxin, another small cytotoxic peptide [59]. However, the authors suggested specific interactions with membranes or proteins

because of the differential effects of KF on the cell membrane in different hepatoma cell lines [59]. Our data clearly point to ErbB3 as a candidate for such specific interaction with KF. In contrast to the anti-tumour activity of EGFR inhibitors, KF-induced cytotoxicity cannot just be explained by targeting a single receptor. Nevertheless, our data may have impact on selecting patients that could benefit from treatment with KF. In addition to this, our data initiated combinations studies of KF and ErbB inhibitors that are currently being evaluated in preclinical studies (J. Jimeno, personal communication).

In conclusion, ErbB targeting agents have shown anti-tumor activity against many types of cancer, including NSCLC. Although much progress has been made in recent years, the data of (prospective) clinical trials with accompanying detailed molecular tumor profile is required to further identify the subpopulation of patients that benefit from this type of therapy. Moreover, more detailed research is needed to improve the efficacy of ErbB targeting agents in combination with chemotherapy, radiotherapy, or other novel (targeted) agents.

References

1. Herbst, RS *et al.* Selective Oral Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor ZD1839 Is Generally Well-Tolerated and Has Activity in Non-Small-Cell Lung Cancer and Other Solid Tumors: Results of a Phase I Trial. *J Clin Oncol* **20**, 3815-3825 (2002).

2. Baselga, J *et al.* Phase I safety, pharmacokinetic, and pharmacodynamic trial of ZD1839, a selective oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with five selected solid tumor types. *J Clin Oncol* **20**, 4292-4302 (2002).

3. Ranson, M *et al.* ZD1839, a selective oral epidermal growth factor receptor-tyrosine kinase inhibitor, is well tolerated and active in patients with solid, malignant tumors: results of a phase I trial. *J Clin Oncol* **20**, 2240-2250 (2002).

4. Gilmore, AP *et al.* Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin-like growth factor receptor. *J Biol Chem* **277**, 27643-27650 (2002).

5. Janmaat, ML *et al.* Response to Epidermal Growth Factor Receptor Inhibitors in Non-Small Cell Lung Cancer Cells: Limited Antiproliferative Effects and Absence of Apoptosis Associated with Persistent Activity of Extracellular Signal-regulated Kinase or Akt Kinase Pathways. *Clin Cancer Res* **9**, 2316-2326 (2003).

6. Moulder, SL *et al.* Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells in vitro and in vivo. *Cancer Res* **61**, 8887-8895 (2001).

7. Wu, X *et al.* Apoptosis induced by an anti-epidermal growth factor receptor monoclonal antibody in a human colorectal carcinoma cell line and its delay by insulin. *J Clin Invest* **95**, 1897-1905 (1995).

8. Liu, B *et al.* Induction of apoptosis and activation of the caspase cascade by anti-EGF receptor monoclonal antibodies in DiFi human colon cancer cells do not involve the c-jun N-terminal kinase activity. *Br J Cancer* **82**, 1991-1999 (2000).

9. Tortora, G *et al.* Cooperative inhibitory effect of novel mixed backbone oligonucleotide targeting protein kinase A in combination with docetaxel and anti-epidermal growth factor-receptor antibody on human breast cancer cell growth. *Clin Cancer Res* **5**, 875-881 (1999).

10. Huang, SM *et al.* Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. *Cancer Res* **59**, 1935-1940 (1999).

11. Sirotnak, FM *et al.* Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* **6**, 4885-4892 (2000).

12. Ciardiello, F *et al.* Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* **6**, 2053-2063 (2000).

13. Paez, JG *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304**, 1497-1500 (2004).

14. Lynch, TJ *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* **350**, 2129-2139 (2004).

15. Pao, W *et al.* EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A* **101**, 13306-13311 (2004).

16. Bell, DW *et al.* Epidermal growth factor receptor mutations and gene amplification in non-small-cell lung cancer: molecular analysis of the IDEAL/INTACT gefitinib trials. *J Clin Oncol* **23**, 8081-8092 (2005).

17. Cappuzzo, F *et al.* Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. *J Natl Cancer Inst* **97**, 643-655 (2005).

18. Tsao, MS *et al.* Erlotinib in lung cancer - molecular and clinical predictors of outcome. *N Engl J Med* **353**, 133-144 (2005).

19. Dei Tos, AP *et al.* Assessing epidermal growth factor receptor expression in tumours: what is the value of current test methods? *Eur J Cancer* **41**, 1383-1392 (2005).

20. Bartlett, JM *et al.* Evaluating HER2 amplification and overexpression in breast cancer. *J Pathol* **195**, 422-428 (2001).

21. Pao, W *et al.* Epidermal growth factor receptor mutations, small-molecule kinase inhibitors, and non-small-cell lung cancer: current knowledge and future directions. *J Clin Oncol* **23**, 2556-2568 (2005).

22. Eberhard, DA *et al.* Mutations in the epidermal growth factor receptor and in KRAS are predictive and prognostic indicators in patients with non-small-cell lung cancer treated with chemotherapy alone and in combination with erlotinib. *J Clin Oncol* **23**, 5900-5909 (2005).

23. Tsuchihashi, Z *et al.* Responsiveness to cetuximab without mutations in EGFR. *N Engl J Med* **353**, 208-209 (2005).

24. Mukohara, T *et al.* Differential effects of gefitinib and cetuximab on non-small-cell lung cancers bearing epidermal growth factor receptor mutations. *J Natl Cancer Inst* **97**, 1185-1194 (2005).

25. Pao, W *et al.* KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med* **2**, e17 (2005).

26. She, QB *et al.* Resistance to gefitinib in PTEN-null HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacologic modulation of constitutive phosphatidylinositol 3'-kinase/Akt pathway signaling. *Clin Cancer Res* **9**, 4340-4346 (2003).

27. Bianco, R *et al.* Loss of PTEN/MMAC1/TEP in EGF receptor-expressing tumor cells counteracts the antitumor action of EGFR tyrosine kinase inhibitors. *Oncogene* **22**, 2812-2822 (2003).

28. Cappuzzo, F *et al.* Akt phosphorylation and gefitinib efficacy in patients with advanced non-small-cell lung cancer. *J Natl Cancer Inst* **96**, 1133-1141 (2004).

29. Tsurutani, J *et al.* Evaluation of two phosphorylation sites improves the prognostic significance of Akt activation in non-small-cell lung cancer tumors. *J Clin Oncol* **24**, 306-314 (2006).

30. Pao, W *et al.* Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* **2**, e73 (2005).

31. Kobayashi, S *et al.* EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* **352**, 786-792 (2005).

32. Toyooka, S *et al.* EGFR mutation and response of lung cancer to gefitinib. *N Engl J Med* **352**, 2136 (2005).

33. Shih, JY *et al.* EGFR mutation conferring primary resistance to gefitinib in non-small-cell lung cancer. *N Engl J Med* **353**, 207-208 (2005).

34. Kobayashi, S *et al.* An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor. *Cancer Res* **65**, 7096-7101 (2005).

35. Carter, TA *et al.* Inhibition of drug-resistant mutants of ABL, KIT, and EGF receptor kinases. *Proc Natl Acad Sci U S A* **102**, 11011-11016 (2005).

36. Baselga, J *et al.* Treating cancer's kinase 'addiction'. *Nat Med* **10**, 786-787 (2004).

37. Deininger, M *et al.* The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood* **105**, 2640-2653 (2005).

38. McKeage, K *et al.* Trastuzumab: a review of its use in the treatment of metastatic breast cancer overexpressing HER2. *Drugs* **62**, 209-243 (2002).

39. Corless, CL *et al.* PDGFRA mutations in gastrointestinal stromal tumors: frequency, spectrum and in vitro sensitivity to imatinib. *J Clin Oncol* **23**, 5357-5364 (2005).

40. Sawaki, A *et al.* Imatinib mesylate acts in metastatic or unresectable gastrointestinal stromal tumor by targeting KIT receptors--a review. *Cancer Chemother Pharmacol* **54 Suppl 1**, S44-S49 (2004).

41. Chen, LL *et al.* A missense mutation in KIT kinase domain 1 correlates with imatinib resistance in gastrointestinal stromal tumors. *Cancer Res* **64**, 5913-5919 (2004).

42. Azam, M *et al.* Mechanisms of autoinhibition and STI-571/imatinib resistance revealed by mutagenesis of BCR-ABL. *Cell* **112**, 831-843 (2003).

43. Kris, MG *et al.* Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA* **290**, 2149-2158 (2003).

44. Fukuoka, M *et al.* Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. *J Clin Oncol* **21**, 2237-2246 (2003).

45. Shepherd, FA *et al.* Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* **353**, 123-132 (2005).

46. Moulder, SL *et al.* A Phase I/II Trial of Trastuzumab and Gefitinib in Patients with Metastatic Breast Cancer That Overexpresses HER2/neu (ErbB-2). *Clin Breast Cancer* **4**, 142-145 (2003).

47. Huang, S *et al.* Dual-agent molecular targeting of the epidermal growth factor receptor (EGFR): combining anti-EGFR antibody with tyrosine kinase inhibitor. *Cancer Res* **64**, 5355-5362 (2004).

48. Matar, P *et al.* Combined epidermal growth factor receptor targeting with the tyrosine kinase inhibitor gefitinib (ZD1839) and the monoclonal antibody cetuximab (IMC-C225): superiority over single-agent receptor targeting. *Clin Cancer Res* **10**, 6487-6501 (2004).

49. Raez, LE *et al.* Clinical responses to gefinitib after failure of treatment with cetuximab in advanced non-small-cell lung cancer. *J Clin Oncol* **23**, 4244-4245 (2005).

50. Faircloth, GT *et al.* Preclinical development of Kahalalide F, a new marine compound selected for clinical studies. *Proc Am Assoc Cancer Res* **41**, 600 (2000).

51. Faircloth, GT *et al.* Selective antitumor activity of Kahalalide F, a marine-derived cyclic depsipeptide. *Proc Am Assoc Cancer Res* **42**, 213 (2001).

52. Medina, LA *et al.* Investigation of the effects of Kahalalide F (PM92102) against tumor specimens taken directly from patients. *Proc Am Assoc Cancer Res* **42**, 213 (2001).

53. Jimeno, J *et al.* Progress in the clinical development of new marine-derived anticancer compounds. *Anticancer Drugs* **15**, 321-329 (2004).

54. Shao, L *et al. In vitro* anti-proliferative effect on sarcoma cells of ET-743 and other marine chemotherapeutics. *Proc Am Assoc Cancer Res* **42**, 203 (2001).

55. Garcia-Rocha, M *et al.* The antitumoral compound Kahalalide F acts on cell lysosomes. *Cancer Lett* **99**, 43-50 (1996).

56. Suarez, Y *et al.* Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells. *Mol Cancer Ther* **2**, 863-872 (2003).

57. Wosikowski, K *et al.* Identification of epidermal growth factor receptor and c-erbB2 pathway inhibitors by correlation with gene expression patterns. *J Natl Cancer Inst* **89**, 1505-1515 (1997).

58. Holbro, T *et al.* The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci U S A* **100**, 8933-8938 (2003).

59. Sewell, JM *et al.* The mechanism of action of Kahalalide F: variable cell permeability in human hepatoma cell lines. *Eur J Cancer* **41**, 1637-1644 (2005).

Chapter 7

Nederlandse samenvatting

Samenvatting

Kanker is een groot probleem in de westerse wereld: in Nederland is het op dit moment de tweede doodsoorzaak na hart en vaatziekten, maar de verwachting is dat het binnen enkele jaren de belangrijkste doodsoorzaak zal worden.

Kanker ontstaat doordat normale cellen in het lichaam ongecontroleerd delen en overleven. In normale weefsels is er een balans tussen cellen die delen en cellen die gecontroleerd doodgaan. De proliferatie en overleving van tumorcellen is daarentegen een ongecontroleerd proces, dat leidt tot een wildgroei van cellen en uiteindelijk het ontstaan van kwaadaardige gezwellen: kanker.

De deling en overleving van normale cellen uit het menselijke lichaam is een nauwgezet en gereguleerd proces, dat wordt geactiveerd door factoren uit de directe omgeving van cellen, zoals hormonen en groeifactoren. Groeifactoren en hormonen kunnen cellen aanzetten tot groei, doordat ze aan receptoren (een soort antennes) binden, die voorkomen op het cel-membraan aan de buitenkant van de cel. Hiermee geven ze een signaal af die de receptoren op hun beurt doorgeven naar de binnenkant van de cel. Hier vindt achtereenvolgens een reeks van chemische reacties plaats (signaal transductie), die uiteindelijk leidt tot activiteit van het DNA in de kern van de cel en deling en overleving van de cel. Aan de andere kant zullen cellen niet overleven als ze beschadigd zijn, een gebrek aan groeifactoren hebben, of een specifiek signaal krijgen en een proces van gecontroleerde celdood (apoptose) in gang zetten.

Opeenhoping van DNA mutaties of veranderingen in de expressie van genen die de groei en overleving van cellen controleren dragen bij aan het ontstaan van kanker. Het is al enige tijd bekend dat ongecontroleerde en verhoogde activiteit van de epidermale groeifactor receptor (EGFR) en gerelateerde receptoren (de ErbB receptoren) belangrijke factoren zijn die bijdragen aan de ontwikkeling van verschillende vormen van epidermale tumoren. Hieronder vallen tumoren van de long, borst, hersenen, en hoofd- en halstumoren. Als gevolg van dit inzicht zijn verschillende middelen ontwikkeld die specifiek aangrijpen op de EGFR en de activiteit van de EGFR remmen. Kleine moleculen die specifiek de enzymatische activiteit van de EGFR remmen (tyrosinekinase remmers), zoals gefitinib (Iressa) en erlotinib (Tarceva), en EGFR-specifieke, monoklonale antistoffen, zoals cetuximab (Erbitux), worden momenteel getest in klinische studies en/of zijn al geregistreerd als antikankermiddel.

Een veelgebruikte therapie tegen kanker is chemotherapie, dat de sneldelende tumorcellen doodt. Helaas worden ook normale delende cellen door chemotherpie gedood, wat zorgt voor een aantal vervelende bijwerkingen (zoals bloedarmoede, vermoeidheid, haarverlies en misselijkheid). Een groot voordeel van EGFR remmers ten opzichte van klassieke chemotherapie, is dat deze middelen veel specifieker op tumorcellen aangrijpen en daardoor minder en mildere bijwerkingen geven. Een nadeel is dat EGFR remmers maar in een klein percentage van de patiënten werken, omdat EGFR maar in een gedeelte van de patiënten verantwoordelijk is voor het ontstaan van hun ziekte.

In dit proefschrift hebben we nader onderzoek gedaan naar de werkingsmechanismen van EGFR remmers. Het doel was om moleculaire eigenschappen te identificeren waaruit kan worden afgeleid of tumor(cell)en gevoelig dan wel resistent zijn voor behandeling met EGFR remmers.

In **hoofdstuk 1** wordt een algemene inleiding gegeven over de EGFR signaal transductie route en de rol hiervan tijdens de embryonale ontwikkeling en in kanker. Ook wordt er een overzicht gegeven van de status van EGFR remmers in preklinische en klinische studies.

In **hoofdstuk 2** onderzoeken we de moleculaire en cellulaire werkings-mechanismen van EGFR remmers in longkankercellen. Na aanleiding van veelbelovende resultaten met de EGFR remmer gefitinib in longkankerpatiënten, waren we geïnteresseerd in de werkingsmechanismen van gefitinib in longkankercellen en hebben dit vergeleken met het EGFR-specifieke antilichaam cetuximab. In tegenstelling tot de resultaten in de kliniek, vonden we dat alle geteste cellijnen ongevoelig waren voor behandeling met EGFR remmers. Deze resistente cellijnen bleken hun intracellulaire signaal transductie onafhankelijk van EGFR te activeren. Deze resultaten suggereren dus dat EGFR-onafhankelijke activatie van intracellulaire signaaltransductie geassocieerd is met resistentie tegen EGFR remmers.

In **hoofdstuk 3** vonden we dat gecombineerde behandeling van gefitinib met specifieke remmers van intracellulaire signaal transductie leidde tot een verminderde celdeling en/of verhoogde celdood van longkankercellen die resistent zijn voor EGFR remmers. Verder vonden we in dit hoofdstuk dat er een aantal longkanker cellijnen ook gevoelig zijn voor behandeling met gefitinib. Deze gevoeligheid bleek te zijn geassocieerd met hoge expressie van EGFR of mutaties in het DNA dat codeert voor de

receptor (*EGFR* gen). Omgekeerd bleken mutaties die voorkomen in het DNA van het intracellulaire signaaltransductie eiwit k-Ras geassocieerd te zijn met resistentie tegen EGFR remmers.

Hoofdstuk 4 beschrijft de resultaten van een klinische studie van gefitinib in eerder behandelde slokdarmkankerpatiënten in een vergevorderd stadium; dit is een groep patiënten die zeer moeilijk te behandelen is. Hoewel er van de 36 beoordeelde patiënten maar één patiënt (2,8 %) een remissie had na behandeling met gefitinib (d.w.z. een tumor die kleiner wordt), had een relatief groot aantal van 27,8 % minimaal een stabiele ziekte tijdens behandeling met gefitinib (d.w.z. een tumor die niet groeit). Vervolgens hebben we van deze groep van patiënten het tumorweefsel bestudeerd voor een aantal biologische kenmerken. Er werden in deze tumoren geen mutaties in het *EGFR* gen, maar het resultaat van patiënten met een hoge expressie van het EGFR eiwit was significant beter. Mutatie in het *k-ras* gen bleken juist voor te komen in patiënten met een voortgaande ziekte tijdens de behandeling met gefitinib. Ook bleken vrouwen en patiënten met een plaveicelcelcarcinoom histologie een significant beter resultaat te hebben.

In **hoofdstuk 5** onderzoeken we de moleculaire en cellulaire werkings-mechanismen van het experimentele antikanker middel kahalalide f (KF). KF bleek op een erg krachtige manier kankercellen te kunnen doden. In tegenstelling tot eerdere publicaties, waarin werd gesuggereerd dat KF de activiteit van EGFR en/of de gerelateerde ErbB2 receptor zou remmen, vonden wij dat KF de expressie van een ander EGFR familielid verlaagt: de ErbB3 receptor. Hierdoor werd ook de intracellulaire signaaltransductie via PI3K-Akt geremd en daarmee de signalen die cellen nodig hebben om te overleven.

Tenslotte worden de resultaten die beschreven zijn in dit proefschrift en hun implicaties in de kliniek besproken in **hoofdstuk 6**. Het lijkt nu duidelijk te worden dat EGFR remmers effectief zijn bij bepaalde vormen van kanker, met name longkanker. Recent onderzoek richt zich op het bepalen van de subpopulatie van patiënten die voordeel heeft bij behandeling met EGFR remmers. Tumorcellen met *EGFR* mutaties, of hoge EGFR expressie zijn gevoelig voor EGFR tyrosinekinase remmers. EGFR-onafhankelijke activiteit van intracellulaire signaaltransductie door ondere andere *k-ras* mutaties is juist geassocieerd met resistentie tegen EGFR remmers.
De verwachting is dat deze ontwikkeling er uiteindelijk toe zal leiden dat de behandeling van kankerpatiënten steeds persoonlijker zal worden.

List of Abbreviations

AD	adenocarcinoma
CDDP	cisplatin
cfu	colony forming units
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Erk	extracellular signal-regulated kinase
FACS	fluorescent activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GSK-3β	glycogen synthase kinase 3β
kDa	kilo Dalton
KF	kahalalide f
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MEK	mitogen-activated protein kinase kinase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NSCLC	non-small cell lung cancer
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PCR	polymarase chain reaction
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
PS	phosphatidyl serine
SCC	squamous cell carcinoma
SD	standard deviation
TGFα	transforming growth factor α
TKI	tyrosine kinase inhibitor
Wt	wild type
YFP	yellow fluorescent protein

Curriculum Vitae

Curriculum Vitae

Maarten Laurens Janmaat werd op 12 september 1976 geboren te Bladel en Netersel. In 1994 behaalde hij zijn VWO diploma aan het Pius-X college in Bladel, waarna hij een jaar aan de Vrije Hogeschool in Zeist het propedeutisch jaar volgde. In 1995 begon hij aan de studie Biologie aan de Universiteit van Utrecht. Tijdens zijn studie rondde hij 2 stages af. Tijdens zijn eerste stage aan de Universiteit van Utrecht bij de vakgroep Moleculaire Celbiologie werkte hij aan de intracellulaire translocatie van Akt/PKB onder begeleiding van Dr. R. Doornbos. Hij voltooide zijn tweede stage aan de Universiteit van Dundee in Schotland onder begeleiding van Prof. C.G. Proud. Tijdens dit project werkte hij aan de regulatie van de activiteit van eIF2B, een eiwit dat betrokken is bij de initiatie van mRNA translatie. Tijdens zijn studie heeft hij meerdere practica moleculaire celbiologie geassisteerd. In augustus 2000 studeerde hij af.

In januari 2001 startte hij zijn promotieonderzoek bij de afdeling Geneeskundige Oncologie aan het VU Medisch Centrum (VUMC) onder begeleiding van Prof. G. Giaccone en Dr. J.A. Rodriguez. Dit project wordt in dit proefschrift beschreven en is getiteld: 'Targeting ErbB receptors as anticancer therapy: Factors of sensitivity and resistance'.

Tijdens de afronding van dit proefschrift is hij begonnen aan een postdoc project bij de afdeling Geneeskundige Oncologie aan het VU Medisch Centrum (VUMC) in de groep van Prof. E. Boven. In dit project bestudeert hij de rol van PDGF signaaltransductie in eierstokkanker.

List of publications

Janmaat ML, Gallegos-Ruiz MI, Rodriguez JA, Meijer GA, Richel D, Van Groeningen C, Giaccone G. Analysis of epidermal growth factor receptor pathway components as predictive factors for response to gefitinib in advanced esophageal cancer patients. Journal of Clinical Oncology 2006, 24: 1612-9.

Janmaat ML, Rodriguez JA, Gallegos-Ruiz MI, Kruyt FA, Giaccone G. Enhanced cytotoxicity induced by gefitinib and specific inhibitors of the Ras or Phosphatidyl Inositol-3 Kinase pathways in Non-Small Cell Lung Cancer cells. International Journal of Cancer 2006, 118: 209-14.

Janmaat ML. Remming van de epidermale groeifactorreceptor bij de behandeling van longkanker. Tijdschrift Kanker 2006, 1: 16-20.

Janmaat ML, Rodriguez JA, Kruyt FA, Giaccone G. Kahalalide F induces necrosis-like cell death that involves depletion of ErbB3 and downstream inhibition of Akt signalling. Molecular Pharmacology 2005, 68: 502-10.

Janmaat ML, Kruyt FA, Rodriguez JA, Giaccone G. Response to epidermal growth factor receptor inhibitors in non-small cell lung cancer cells: limited antiproliferative effects and absence of apoptosis associated with persistent activity of extracellular signal-regulated kinase or Akt kinase pathways. Clinical Cancer Research 2003, 9: 2316-26.

Janmaat ML, Giaccone G. The epidermal growth factor receptor pathway and its inhibition as anticancer therapy. Drugs of Today 2003, 61 (Suppl. C): 61-80.

Janmaat ML, Giaccone G. Small-molecule epidermal growth factor receptor tyrosine kinase inhibitors. The Oncologist 2003, 8: 576-86.

Wang X, **Janmaat M**, Beugnet A, Paulin FEM, Proud CG. Evidendence that the dephosphorylation of Ser535 in the e-subunit of eukaryotic initiation factor (eIF) 2B is insufficient for the activation of eIF2B by insulin. Biochemical Journal 2002, 367: 475-81.

Dankwoord

Dankwoord

Iemand stapt in een rivier, de stroom brengt hem naar de andere oever en daar stapt hij weer aan wal. Dan buigt hij zich naar de rivier en bedankt de stroom van het water. Voor de rivier is het om het even. Dat is danken.
Bert Hellinger

Ik wil graag iedereen bedanken die direct of indirect betrokken is geweest bij het schrijven van dit boekje; een aantal mensen wil ik hier met name noemen.

Promotor Prof. Giaccone, Beppe, bedankt dat je als autoriteit op het gebied van EGFR remmers in de kliniek mij de kans en het vertrouwen hebt gegeven om als AiO aan dit project te werken in het lab. Jouw begeleiding stimuleerde me om vaak tot het uiterste te gaan, wat uiteindelijk heeft geleid tot dit mooie eindresultaat.
Co-promotor Dr. Rodriguez, Josean, thank you for all the things you have taught me about science, ranging from practical work to discussing, presenting, and writing down results. Your humor, advises and pragmatism were always refreshing and helpful.

Natuurlijk wil ik ook de leden van de leescommissie en promotiecommissie bedanken voor de aandacht die ze aan dit proefschrift hebben besteed. Prof. Gerrit Meijer en Dr. Cees van Groeningen wil ik ook bedanken voor hun bijdrage aan hoofdstuk 4.

Iedereen van de apoptose-groep; de mensen van het eerste uur: Frank, Simone (ik zou willen dat ik iets van jouw organisatietalent had), Carlos, Miriam, Linda, Cynthia, en de mensen die (wat) later kwamen: Barbara (thanks for the nice chats in our room), Agnieszka (good luck in the US!), Mariëlle (eerst analist, nu mijn 'opvolgster', succes met jouw onderzoek!), Jens, Bas, Karijn, bedankt voor alle hulp en gezelligheid binnen en buiten het lab!

Alle studenten die voor langere of kortere tijd aan dit onderzoek hebben meegewerkt: Annemiek, Federico, en Ilaria.

De onco mensen “van de overkant”, met name M-A-R-C-E-L en D-E-N-N-I-S (lingo!).
Marcel, ik vind het geweldig dat jij paranimf wilt zijn bij mijn promotie! Jouw enthousiasme, humor en muziekkennis waardeer ik enorm. Ondanks dat je nu helemaal in Enschede woont, ben ik ervan overtuigd dat we elkaar nog regelmatig zullen tegenkomen in de toekomst!
Alle andere mensen van oncologie: Epie, Maaike, Frits, Chiel (monumental), Kees, Henk D, en alle anderen voor hun hulp, advies, en gezelligheid.
De secretaresses Sandra en Monique voor hun administratieve advies en ondersteuning tijdens het schrijven van dit boekje.

Het squash-clubje, waarmee ik wekelijks mijn energie kwijt kon; Sander, Saskia, Bart, Stefan, Olaf, Laura en Pim. Squash en de sessies in het DE café met Saskia en Marcel waren onmisbaar om stoom af te blazen in stressvolle tijden. Sas, mede-directeur, succes met de afronding van jouw promotie; nog even volhouden!

Ook wil ik graag een aantal mensen buiten het lab bedanken.
Als eerste paranimf Laurens: vanaf de eerste dag van de a-dagen ’95 een hele goeie vriend, dank je voor alle interesse, goeie gesprekken en squash! Maar ook alle andere vrienden die tijdens mijn AiO-tijd voor de broodnodige afleiding zorgden: Bas, Jurriën, en Marcel van B., voor alle gezellige poker/whiskey/BBQ avonden; Barbara, voor haar belangstelling, etentjes, en koffiedrinksessies (we moeten weer een keer muziek maken!); Jeroen, voor de geweldige strijd op de squashbaan; en alle anderen.

Mijn (schoon)familie: Ria, Anton, Miriam, Casper, Lola, Simone, Edwin, Tim, Jasper, Gerard, Erna, Marjon, en Michiel, bedankt voor jullie support en belangstelling. Mam, heel erg bedankt voor alle kansen die je me hebt gegeven!

In het bijzonder wil ik het thuisfront bedanken. Karin en Julie, aan jullie draag ik mijn boekje op. Lieve Karin, dank je voor je steun, liefde en vriendschap, bij jou ben ik echt thuis. Julie, als je lacht ben ik volmaakt gelukkig, dankjewel lieve meid.

Eindelijk is ‘ie af, bedankt allemaal!

Maarten